Mechanisms underlying the endothelium-dependent modulation of vascular tone

Iarova, Polina

Award date:
2011

Awarding institution:
University of Bath

Mechanisms underlying the endothelium-dependent modulation of vascular tone

Polina Iarova (Yarova)

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

August 2011

Supervisors:

Dr. Kim Dora

Prof. Chris Garland

Dr. Sergey Smirnov

Dr. Roland Jones

COPYRIGHT

SIGNED……..

”Discovery is about exposing the mysteries of the nature, of what already exists. Invention is about creating something that never existed before (…) Discovery is about the excitement of travelling. Invention is about satisfaction of arriving when it becomes ‘fit for purpose’.”

Sir James Whyte Black

Abstract

1. Vascular tone is a key determinant of blood pressure and tissue blood flow. In this study I investigated local and distant modulation of arterial tone by the endothelium during activation of endothelium-dependent and -independent signalling pathways.
2. The results obtained confirm that in rat small mesenteric arteries the endothelium modulates the tone of the underlying smooth muscle cells (SMCs) via release of relaxing and contracting factors (EDRFs and EDCFs, respectively). EDRF involved signalling of nitric oxide synthase (NOS) that produced nitric oxide (NO) in two forms: free radical ($NO^{\bullet}$) and nitroxyl anion (NO^{-}). The latter but not the former stimulated both big conductance Ca^{2+}-activated potassium (BK_{Ca}) and voltage-gated potassium (K_v) channels via guanylyl cyclase signalling and was able to evoke conducted dilatation.
3. Physiological adrenergic agonists, adrenaline or noradrenaline (NA), suppressed local dilatation to the endothelium-dependent agonist acetylcholine (ACh). The main role belonged to β-adrenoceptors, which via cAMP signalling suppressed NO signalling, probably by inhibiting eNOS, and intermediate conductance Ca^{2+}-activated potassium (IK_{Ca}) channel components of the dilatation, whilst enhancing the conducted response.
4. The endothelium did not directly participate in the local dilatation to β-adrenoceptor stimulation by release of EDRFs. Rather it served to create a semi-permeable barrier that reduced the potency of luminal adrenergic agonists to act on the underlying smooth muscle cells. Moreover, stimulation of β-adrenergic receptors, especially in old and hypertensive animals, was associated with endothelial cell COX signalling, which attenuated the local dilatation.

5. β-adrenoceptors were also able to evoke conducted dilatation that spread along the vessel wall in a similar way to the dilatation evoked by ACh. Smooth muscle K_{ATP} channels did not contribute much to the local response, but were essential for the conducted dilatation. The conducted response to both agonists was enhanced by stimulation with a thromboxane (TP) receptor agonist.
6. Taken together, this study demonstrates that the endothelium does not directly participate in β-adrenoceptor-mediated vasodilatation, and stimulation of the receptor leads to the inhibition of endothelium-derived dilatation. However, the endothelium appears crucial for establishing conducted vasodilatation, providing a pathway for the hyperpolarization evoked locally by either β-adrenergic stimulation or endogenous NO^- production.

Acknowledgements

I would like to thank my supervisors Dr Kim Dora, Prof. Christopher Garland, Dr Sergey V. Smirnov and Dr. Roland Jones for the opportunity to perform my study in the University of Bath, their highly appreciated advice and support during my work. I am particularly grateful to Dr Kim Dora and Prof. Christopher Garland for introducing me to the exiting field of vascular pharmacology, teaching me to high standard and with novel methods, help, guidance and encouragement during my study. I also appreciate the opportunities given to me to present my work at meetings in the UK and overseas, which allowed me to broaden my mind, share ideas and get feedback from colleagues around the world.

I owe a big thank you to all my friends and colleagues in the vascular pharmacology research group for their kind help and friendship, particularly to Dr Timea Beleznai, Dr Kathryn Yuill, Dr Rosalia Rodriguez and Dr Francesc Jimenez-Altayo, fruitful collaboration with whom led to our joint publications. I acknowledge the very useful feedback, advice and interesting ideas given to me by Dr Amanda MacKenzie, Dr Chris Bailey and Chloe Lim, which improved the work presented here. And finally, without understanding, instant support and personal sacrifice of my family completion of this work would not have been possible to perform; I give especial hearty gratitude to them.

The work was funded by University of Bath and ORS scholarship.

Published work arising from this thesis

Published articles:

Yuill K, Yarova P, Kemp-Harper B, Garland C, Dora K "A novel role for HNO in local and spreading vasodilatation in rat mesenteric resistance arteries" // *Antioxid Redox Signal*. 2010 Jul 8.

C.J. Garland, P.L. Yarova, F. Jimenez-Altayo and K.A. Dora "Vascular hyperpolarization to β-adrenoceptor agonists evokes spreading dilatation in rat isolated mesenteric arteries" // *Br J Pharmacol*. 2011 Jan 11.

Timea Z. Beleznai, Polina L. Yarova, Kathryn H. Yuill and Kim A. Dora "Smooth muscle Ca^{2+}-activated and voltage-gated K^+ channels modulate conducted dilation in rat isolated small mesenteric arteries" // *Microcirculation.* 2011 Apr 27.

Abstracts presented on conferences:

P.L. Yarova, C.J. Garland, K.A. Dora "Negative cross-talk between endothelial M_3-muscarinic and β-adrenoceptors in rat resistance mesenteric arteries" // *Young Physiologists' Symposium*, Ion Channels and Receptors in Cell Physiology, University of Leicester, Leicester, 2009.

P.L. Yarova, C.J. Garland, K.A. Dora "β-adrenergic receptors differentially modulate local and spreading vasodilatation evoked by endothelial cell muscarinic receptors in rat pressurized mesenteric arteries" // *BPS Winter Meeting*, Queen Elizabeth II Conference Centre, London, 2009.

PL. Yarova, KA. Dora, CJ. Garland "Activation of β-adrenoceptors evokes local and spreading dilatation in rat isolated mesenteric arteries" // *60th Annual British Microcirculation Meeting*, Peninsula Medical School, Exeter, 2010.

PL. Yarova, CJ. Garland, KA. Dora "Vasodilatation to NO^- in rat small mesenteric arteries; a role for potassium channels" // *WorldPharma 2010*, 16th IUPHAR World Congress of Basic and Clinical Pharmacology, Copenhagen, 2010.

Polina Yarova, Chris Garland, Kim Dora "A novel role for endogenous nitroxyl anion in local and spreading vasodilatation in rat small resistance arteries" // *Oxford BHF CRE Annual Symposium*, BHF Centre of Research Excellence, Said Business School, Oxford, 2010.

P.L. Yarova, C.J. Garland, K.A. Dora "The agonist used to generate tone affects the decay of spreading vasodilatation in rat mesenteric arteries" // *9th World Congress for Microcirculation*, Maison de la Chimie, Paris, 2010.

PL. Yarova, KA. Dora, CJ. Garland "A role for β-adrenoceptors in spreading vasodilatation" // *Experimental Biology 2011,* Walter E. Washington Convention Center, Washington, DC, 2011.

Table of Contents

List of Figures

List of Abbreviations

ACh, acetylcholine; ATP, adenosine 5'-triphosphate; BIS-I, bisindolylmaleimide I; cAMP, 3'-5'-cyclic adenosine monophosphate; $[cAMP]_i$, intracellular cAMP levels; cGMP, 3'-5'-cyclic guanosine monophosphate; CRC, concentration-response curves; DAG, diacylglycerol; DMSO, dimethylsulfoxide; EC, endothelial cell; EETs, epoxyeicosatrienoic acids; EDH, endothelium-derived hyperpolarization; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; e, endothelial; ER, endoplasmic reticulum; GDP, guanosine diphosphate; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; H_2O_2, hydrogen peroxide; i, inducible; IEL, internal elastic lamina; IP_3, inositol 1,4,5-triphosphate; K^+, potassium ion; K, kinase; K_{ATP}, ATP-sensitive K^+ channel; K_{Ca}, Ca^{2+}-activated K^+ channel (B, big; I, intermediate; S, small conductance); K_{ir}, inwardly rectifying K^+ channel; K_v, voltage-gated K^+ channel; L-NAME, N^{ω}-nitro-L-arginine methyl ester hydrochloride; L_{Ca} channel, L-type voltage-gated Ca^{2+} channel; NA, noradrenaline; MEGJ, myoendothelial gap junction; MLC, myosin light chain; MOPS, 3-[N-morpholino]propane-sulfonic acid; n, neuronal; Na^+, sodium; NCX, Na^+-Ca^{2+} exchanger; NO, nitric oxide; $NO^{\bullet}$-uncharged free radical state of NO; NO^+ - nitrosonium cation; NO^-, nitroxyl anion; NOS, nitric oxide synthase; Oregon Green BAPTA-1 AM, Oregon Green 488 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetate; PE, phenylephrine; PKA, c-AMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PLA, phospholipase A; PLC, phospholipase C; RyR, ryanodine receptor, SMC, smooth muscle cell; SOCE, store-operated Ca^{2+} entry; T_{Ca}, T-type voltage-gated Ca^{2+} channel; TP, thromboxane (receptor); TRP, transient receptor potential channel; TRPC, canonical TRP; TRPM, melastatin TRP; TRPV, vallinoid TRP; STIM-1, stromal interacting molecule 1.

Chapter 1. Introduction

1.1 Principles of blood flow development and structure of resistance arteries

Live tissue metabolism requires a continual supply of oxygen and nutrients in the amounts sufficient to cover its current needs. The ultimate source of such nutrients is the blood, transported by arteries feeding the tissue. The blood flow is driven by the difference in the arterial and venal pressures created by the cardiac output and vascular resistance to the flowing blood. This can be expressed as (Levy, 1979):

$$Q = \frac{\Delta P}{R}, \qquad \text{(Equation 1.1)}$$

where Q is the rate of blood flow, ΔP is the pressure gradient and R is the resistance of the artery. Vascular resistance occurs due to the friction of the passing blood against the vessel wall, and can be calculated by means of the modified Hagen-Poiseuille equation:

$$R = \frac{128 \mu l}{\pi d^4}, \qquad \text{(Equation 1.2)}$$

where μ is viscosity, l is vessel length and d is the vessel diameter. Therefore the blood flow can be determined as:

$$Q = \frac{\Delta P \pi d^4}{128 \mu l}, \qquad \text{(Equation 1.3)}$$

It can be seen from the equation that the blood flow depends on the arterial diameter raised to the power of four. Such a high dependency provides an important mechanism of regulation of the circulation by a fine modulation of the arterial diameter with numerous physicochemical stimuli. Small resistance arteries, in contrast to the big conduit vessels, are able to considerably change their diameter, thus enabling the fine control of blood pressure and tissue blood flow.

Due to the large amount of blood that flows through the mesenteric artery, this vascular bed significantly contributes to the regulation of arterial pressure. Mesenteric arteries being a part of the splanchnic circulation system supply blood to the duodenum and transverse colon. The main (superior) mesenteric artery arises from the abdominal aorta, then separates to multiple branches and forms a mesenteric arcade (Figure 2.1B). Small mesenteric arteries from the arcade are recognised as resistance arteries. Their wall, as with all blood vessels, consists of three main layers: tunica adventitia, tunica media and tunica intima (Mulvany *et al.*, 1990). The tunica adventitia is the outer layer of the arterial wall consisting of collagen and elastic fibres, mast cells, macrophages, fibroblasts and small arterioles that feed the vessel wall. It is also laced with axons of sympathetic and sensory neurons, and adventitial neuronal cell bodies containing calcitonin gene-related peptide located in close proximity to the sensory nerve fibres (Somasundaram *et al.*, 2006). Tunica media consists of several layers of thin, long smooth muscle cells that go around the arterial lumen, and cells with irregular shape and long filaments called "interstitial Cajal-like cells" (Formey *et al.*, 2011). Lining the internal side of the vessel, the tunica intima consists of a single layer of longitudinal endothelial cells. Between the tunica intima and tunica media there is an internal elastic lamina, a thin layer of elastic fibres with holes in it. Endothelial cells may form projections which reach smooth muscle cells through these holes to create myo-endothelial gap junctions, allowing direct cell-to-cell communication (Rhodin, 1967; Sandow *et al.*, 2000).

1.2 Contraction and relaxation of vascular smooth muscle

The ability of vascular smooth muscle cells to contract and relax in response to certain stimuli underlies the main mechanism of arterial lumen diameter regulation. The ignition which initializes the force generation in smooth muscle cell is a rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Regardless of the source, Ca^{2+} binds to calmodulin and leads to the calmodulin-dependent activation of myosin light chain (MLC) kinase. The phosphorylation of MLC stimulates myosin ATPase, promoting hydrolysis of adenosine-5'-triphosphate (ATP) and formation of cross-bridges between myosin and actin filaments (Dillon *et al.*, 1981; Hai *et al.*, 1989). MLC phosphatase dephosphorylates MLC leading to smooth muscle relaxation. Therefore, control of vascular tone mainly occurs by means of modulation of the smooth muscle cell $[Ca^{2+}]_i$ or by altering the sensitivity of contractile filaments to Ca^{2+} via regulation of MLC kinase and phosphatase activity (Ratz *et al.*, 2005).

1.2.1 Voltage-gated Ca^{2+} channels

One of the main sources of Ca^{2+} during initiation of smooth muscle contraction is extracellular Ca^{2+} ($[Ca^{2+}]_o$), which enters the smooth muscle cell via voltage-gated Ca^{2+} channels upon depolarization of the cell membrane in response, for example, to raised extracellular potassium concentration $[K^+]_o$ or $G_{q/11}$-protein coupled receptor signalling (Nelson *et al.*, 1988). In contrast, hyperpolarization of the cell membrane will result in the closure of voltage-gated Ca^{2+} channels, reduction of $[Ca^{2+}]_i$ and, subsequently, dilatation (Jaggar *et al.*, 1998). The channel family includes ten members, with two of them found in the vasculature. The L-type voltage-gated Ca^{2+} channel (L_{Ca} channel) produces current that requires high depolarization in order to be activated (between -10mV and -30mV depending on L_{Ca} channel subtype), whilst current through the T-type

voltage-dependent Ca^{2+} channel (T_{Ca} channel) is activated in lower membrane potential (around -45 mV). Current through these channels slowly decayes upon depolarization beyond -40 mV and reverses between +40 and +50 mV (Catterall *et al.*, 2005; Smirnov *et al.*, 1992). The major role of L_{Ca} channels in blood pressure maintenance was shown using mice globally lacking L_{Ca} channels. In this mouse model, the mean blood pressure was decreased by a quarter and the pressor response to $G_{q/11}$-protein coupled receptor stimulation was severely reduced (Moosmang *et al.*, 2003). Comparable effects in rats were evoked by pharmacological blockage of L_{Ca} channels (Pinterova *et al.*, 2010). It is generally assumed also that L_{Ca} channels are responsible for the vasoconstriction in response to depolarization in rat mesenteric arteries (Ball *et al.*, 2009; Leung *et al.*, 2010; Rahman *et al.*, 2007); the role of T_{Ca} channels was shown to increase with decrease of arterial size (Ball *et al.*, 2009; Jensen *et al.*, 2004).

1.2.2 $G_{q/11}$ protein-coupled receptor signalling

The majority of vasoconstrictors, such as adrenaline, thromboxane A_2, and endothelin-1, mediate their action via stimulation of their respective seven-transmembrane domain receptor coupled to heteromeric $G_{q/11}$ protein. Upon activation, the receptor acts as a guanine nucleotide exchange factor, promoting exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) in the α subunit of the G protein, which leads to dissociation of the α subunit from the βγ subunit (Clapham *et al.*, 1997). When dissociated, the α subunit activates phospholipase C (PLC) β_1 that catalyzes phosphatidylinositol-4,5-biphosphate (PIP_2) breakdown, leading to production of inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Berridge *et al.*, 1989). IP_3 activates IP_3 receptors located on the sarcoplasmic reticulum and causes Ca^{2+} efflux from the intracellular stores (Grayson *et al.*, 2004). This initial rise in $[Ca^{2+}]_i$ can evoke further release of Ca^{2+} from the intracellular store via stimulation of ryanodine

receptors (so-called 'Ca^{2+}-induced Ca^{2+} release'), which contributes to the global $[Ca^{2+}]_i$ and facilitates the pace of Ca^{2+} wave propagation within the cell (Boittin *et al.*, 1999).

An additional pathway for a $[Ca^{2+}]_i$-dependent rise of $[Ca^{2+}]_i$ may occur via opening of voltage-gated Ca^{2+} channels triggered by depolarization. Firstly, the release of Ca^{2+} from intracellular stores could stimulate Ca^{2+}-sensitive chloride channels (Akata *et al.*, 2003; Piper *et al.*, 2004), which evokes efflux of negative charged ions that depolarize the smooth muscle cell membrane (Leblanc *et al.*, 2005). Secondly, intracellular Ca^{2+} may also activate the forward mode of the Na^+/Ca^{2+} exchanger (NCX) to extrude one Ca^{2+} ion in return for three Na^+ ions (Berra-Romani *et al.*, 2010; Horiguchi *et al.*, 2001; Weiss *et al.*, 1993), which also results in cation influx and membrane depolarization.

Another product of PLC activity, DAG, can also contribute to depolarization and increase of $[Ca^{2+}]_i$ by activation of non-selective cation current through canonical transient receptor potential (TRPC) channels (Gudermann *et al.*, 2004; Hardie, 2007). Rat mesenteric arterial myocytes appear to express three types of TRPC channels: TRPC1, TRPC4 and TRPC6 (Brueggemann *et al.*, 2006; Hill *et al.*, 2006), and since only TRPC2, TRPC3, TRPC6, and TRPC7 display sensitivity to DAG (Gudermann *et al.*, 2004; Hardie, 2007; Large *et al.*, 2009), it seems the TRPC6 channel subtype is responsible for the effects of DAG in rat mesenteric arteries. In accordance to this it was recently demonstrated that a DAG analogue 1-oleoyl-2-acetyl-sn-glycerol (OAG) evoked whole-cell cation currents mainly via TRPC6 channel in rabbit mesenteric arterial myocytes (Albert *et al.*, 2008).

Lastly, the βγ subunit of G-protein is also known to be an important intracellular effector that modulates function of Ca^{2+} and potassium (K^+) channels as well as phospholipases A and C (Clapham *et al.*, 1997), although its role in the modulation of smooth muscle tone during $G_{q/11}$ protein-coupled receptor signaling is to be determined.

1.2.3 Store-operated Ca^{2+} entry

Contraction of smooth muscle in precapillary arterioles in response to $G_{q/11}$-coupled receptor stimulation does not always depend on extracellular Ca^{2+}, but rather on Ca^{2+} release from the from sarcoplasmic reticulum and intence Ca^{2+} re-uptacke by the SERCA (Borisova *et al.*, 2009). In small arteries, however, Ca^{2+} released from intracellular stores is responsible for the initial contraction, and, if extracellular Ca^{2+} is unavailable, the responses to contractile stimuli appear to be transient (Lagaud *et al.*, 1999; Lamont *et al.*, 2004). This occurs partly due to absence of Ca^{2+} efflux via L_{Ca} channels and due to extrusion of Ca^{2+} from the cell by NCX (Berra-Romani *et al.*, 2010; Horiguchi *et al.*, 2001; Weiss *et al.*, 1993) or by plasma membrane Ca^{2+}-ATPase (Gros *et al.*, 2003); however, the main reason seems to be the limited capacitance of the intracellular stores, which may be easily depleted and can not serve as a sustained Ca^{2+} source to maintain prolonged contraction (Berridge, 1995; Lagaud *et al.*, 1999).

Store-operated Ca^{2+} entry (SOCE), or capacitative Ca^{2+} entry, is a response of the cell to depletion of the intracellular stores, which appears as activation of a non-selective cation channel permeable to Ca^{2+} (Berridge, 1995). For a long time the channel responsible for the capacitative Ca^{2+} entry was not determined (Berridge, 1995), however now TRPC channel is recognized as the main candidate for the store-operated channel (Nilius, 2004). Among proposed channel subtypes responsible for SOCE in vascular smooth muscle cells TRPC1, TRPC4, and TRPC5 are present (Beech *et al.*, 2004; Xu *et al.*, 2001; Xu *et al.*, 2006), with the TRPC1 subtype seemingly playing the main role in rat mesenteric artery smooth muscle cells (Brueggemann *et al.*, 2006).

In arterial myocytes, SOCE channels were found to be regulated by two transmembrane proteins, STIM1 (stromal interacting molecule 1) and Orai1 (the Ca^{2+}

release-activated Ca^{2+} channel modulator) (Baryshnikov *et al.*, 2009). It was demonstrated that upon depletion of the intracellular stores STIM1 translocates from the endoplasmic reticulum to the plasma membrane, functioning as a Ca^{2+} sensor and a link between store depletion and SOCE (Zhang *et al.*, 2005). Orai1 may form the Ca^{2+} selectivity filter of the SOCE channel. This was demonstrated by a point mutation that resulted in change of selectivity properties of SOCE channel from being Ca^{2+}-selective with inward rectification to being selective for monovalent cations and outwardly rectifying (Yeromin *et al.*, 2006). The role of STIM1 and Orai1 in modulation of tone of rat small mesenteric arteries has not been studied yet; however, these molecules were shown to participate in Ca^{2+} handling during growth and remodelling of this tissue (Berra-Romani *et al.*, 2008).

Depletion of intracellular stores may also stimulate further Ca^{2+} influx by means of activation of NCX in reverse mode that extrudes three Na^{+} in exchange for one Ca^{2+} (Baryshnikov *et al.*, 2009; Davis *et al.*, 2009). NCX is spatially co-expressed with Orai1 in the plasma membrane of proliferating human arterial myocytes and displays a functional association with SOCE (Baryshnikov *et al.*, 2009). Functional and spatial association between NCX and SERCA was also shown for pig coronary artery smooth muscle cells (Davis *et al.*, 2009). In rat mesenteric arteries, NCX was also demonstrated to participate in the Ca^{2+} entry after the store depletion (Lagaud *et al.*, 1999), but the exact mechanisms are yet to be determined.

1.2.4 Ca^{2+} sensitization mechanism

Although signalling by vasoconstrictors acting through receptors coupled to $G_{q/11}$ proteins evoke membrane depolarization, rises in $[Ca^{2+}]_i$ and contraction via mechanisms described earlier (see Section 1.2.1), the rise in $[Ca^{2+}]_i$ associated with $G_{q/11}$ protein signalling leads to a greater force development than Ca^{2+} influx achieved in

response to depolarization alone (Bradley *et al.*, 1987; Mulvany *et al.*, 1982). This occurs due to so-called 'Ca^{2+}-sensitization' that mainly involves activation of MLC kinase and/or inhibition of MLC phosphatase activity resulting in greater efficacy of Ca^{2+} (Ratz *et al.*, 2005; Somlyo *et al.*, 2003).

Two major pathways have been identified in rat mesenteric arteries. The first pathway involves activation of protein kinase C (PKC) by signalling of receptors coupled to $G_{q/11}$ protein (Budzyn *et al.*, 2006). The pathway may involve DAG, as DAG was shown to be involved in activation of PKC and consequent Ca^{2+} sensitization in rat cerebral arteries (Gokina *et al.*, 1999). One of the main mechanisms implicated in PKC-induced Ca^{2+} sensitization involves activation of PKC-potentiated inhibitor protein of 17 kDa (CPI-17) that suppress MLC phosphatase (Eto *et al.*, 1997). Another mechanism involves phosphorylation of calponin, a thin filament-associated protein that in basal conditions suppresses the binding between actin and myosin; when calponin is phosphorylated by PKC, it changes its conformation and promotes the contraction (Pohl *et al.*, 1997).

The second pathway involves another serine-threonine kinase, Rho-kinase, which is activated by α subunits of $G_{q/11}$ and $G_{12/13}$ proteins via small GTPase RhoA. Rho-kinase downregulates the activity of MLC phosphatase, resulting in enhanced force production for given $[Ca^{2+}]_i$ (Ratz *et al.*, 2005; Somlyo *et al.*, 2003). The signalling pathway in rat arteries may also involve CPI-17 (Freitas *et al.*, 2009). Rho-kinase is implicated in the generation of tone in rat mesenteric arteries and veins in response to a rises in intramural pressure (Enouri *et al.*, 2010; VanBavel *et al.*, 2001). It also participates in contraction of larger arteries, such as aorta or superior mesenteric artery, in response to α-adrenergic or TP receptor stimulation, or application of high K^+ (Budzyn *et al.*, 2006). Involvement of Rho-kinase in Ca^{2+} sensitization in response to

contractile agonists in small mesenteric arteries is subject to debate. Some studies have shown that small mesenteric arteries lack the Rho-kinase-dependent component of the contraction to the above stimulus (Budzyn *et al.*, 2006), whilst others demonstrate a role for Rho-kinase in the contraction to α-adrenergic agonist (VanBavel *et al.*, 2001).

1.2.5 G_s and $G_{i/o}$ protein-coupled receptors

Receptors coupled to G_s or $G_{i/o}$ proteins, respectively stimulate or inhibit adenylyl cyclase, the enzyme that produces cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). In vascular tissues, cAMP can directly activate cyclic nucleotide-gated ion channels (Leung *et al.*, 2010; Shen *et al.*, 2008) as well as act on two distinct effector proteins: protein kinase A (PKA) (Simonds, 1999) and exchange proteins directly activated by cAMP (Epac) (Purves *et al.*, 2009).

Cyclic nucleotide-gated channels are nonselective cation channels that belong to the voltage-gated ion channel family and are opened by the direct binding of cyclic nucleotides, cAMP and cGMP (Kaupp *et al.*, 2002). Cyclic nucleotide-gated channels were shown to be involved in adrenaline-mediated rises in $[Ca^{2+}]_i$ in cultured endothelial cells from bovine aorta (Shen *et al.*, 2008). These channels were shown to be expressed in rat mesenteric artery smooth muscle (Kruse *et al.*, 2006), where they may contribute to contraction mediated by the TP receptor agonist U46619 (Leung *et al.*, 2010).

PKA has diverse signalling cascades. On the one hand, PKA phosphorylates and inactivates MLC kinase (Conti *et al.*, 1981), on the other hand, it reduces Ca^{2+} entry via L_{Ca} channels by opening ATP-sensitive K^+ (K_{ATP}) channels (Quinn *et al.*, 2004; Shi *et al.*, 2007), voltage-gated K^+ (K_v) channels (Aiello *et al.*, 1998; Waldron *et al.*, 1999), and big conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channels (Tian *et al.*, 2004). In addition, PKA causes direct inhibition of store-operated (Liu *et al.*, 2005) and L_{Ca} channels (Liu

et al., 1997; Orlov *et al.*, 1996; Xiong *et al.*, 1994). All these effects result in hyperpolarization, decrease in smooth muscle cell $[Ca^{2+}]_i$ and relaxation. On the other hand, PKA has been also shown to activate PKC (Wooten *et al.*, 1996), potentially leading to Ca^{2+} sensitization in smooth muscle cells, although this evidence has not received wide acclaim.

Finally, cAMP signalling involves stimulation of Epac, a guanine nucleotide exchange factor for the small G protein Rap (Bos, 2006; Bos, 2003; Gloerich *et al.*, 2010), which was shown to reduce K_{ATP} channel activity in rat aortic smooth muscle cells (Purves *et al.*, 2009). Markedly, it was reported that Epac is able to stimulate PLC in cultured human embryonic kidney cells (Schmidt *et al.*, 2001), although whether it exhibits similar actions in small resistance arteries has yet to be confirmed.

1.2.5 K^+ channels in vascular smooth muscle cells

K^+ channels largely contribute to control of smooth muscle cell membrane potential. Outward K^+ current through the majority of K^+ channels results in cell membrane hyperpolarization, which leads to closure of voltage-gated Ca^{2+} channels and vasodilatation. Some K^+ channels may cause influx of K^+ into the cell; therefore fine control of vascular tone is also enabled by modulation of K^+ channel conductivity (Ko *et al.*, 2008).

One of the main K^+ channels responsible for maintenance of the smooth muscle cell membrane potential is the delayed rectifier K_v channel (Ko *et al.*, 2008; Nelson *et al.*, 1995). The channel consists of a pore-forming α-subunit and regulatory β-subunit, and activates when depolarization reaches to approx. -40 mV with a single channel conductivity of ~10 pS (Ko *et al.*, 2008; Nelson *et al.*, 1995; Shibasaki, 1987). The K_v channel is regulated by PKA, PKC and PKG (Ko *et al.*, 2010), and is sensitive to intracellular magnesium concentration (Tammaro *et al.*, 2005). In mesenteric arteries,

K_v channels are expressed in smooth muscle cells (Xu *et al.*, 1999), where they participate in hyperpolarization in response to nitroxyl anion (Irvine *et al.*, 2003a; Yuill *et al.*, 2010).

Although a rise in $[Ca^{2+}]_i$ is generally associated with contraction, it can also activate an outward K^+ current via BK_{Ca} channels (Xia *et al.*, 2002). The channel is also sensitive to membrane potential, and, as in other K_v channels, the voltage sensor is located in a pore-forming α-subunit, whilst the β-subunit provides the Ca^{2+}-sensitivity of the BK_{Ca} channel (Ko *et al.*, 2008). In the presence of high $[Ca^{2+}]_i$ (starting from 500 nM), the channel open probability is increased, enabling outward K^+ current of 200-250 pS (Nelson *et al.*, 1995; Xia *et al.*, 2002). BK_{Ca} channel is coupled to ryanodine receptors and is implicated in Ca^{2+} spark-induced membrane hyperpolarization in arteries (Jaggar *et al.*, 1998) or urinary bladder (Petkov *et al.*, 2005). Moreover, BK_{Ca} channels were found to form a complex with vanilloid TRP (TRPV) channels, enabling relaxation of cerebral arteries to endothelium-derived epoxyeicosatrienoic acids (EETs) (Earley *et al.*, 2005).

Less abundant is an inward-rectifying K^+ (K_{ir}) channel, which is characterized by conduction of inward current at membrane potentials negative to the equilibrium potential and smaller outward currents at membrane potentials positive to equilibrium potential (Nelson *et al.*, 1995). Therefore, the activity of K_{ir} channels provide a pathway by which changes in extracellular K^+ can alter smooth muscle membrane potential (Quayle *et al.*, 1997). This mechanism plays a crucial role in endothelium-derived hyperpolarization (Dora *et al.*, 2008; Edwards *et al.*, 1998).

Sarcoplasmic K_{ATP} channel is a hetero-octamer, which consists of four K_{ir} channels and four sulphonylurea receptors (SURs). Decreasing levels of intracellular ATP allow the K_{ATP} channel to open with a conductivity of 50-250 pS in rat vascular

smooth muscle cells (Ko *et al.*, 2008). The K_{ATP} channel is responsible for hyperpolarization in response to β-adrenoceptor stimulation (Garland *et al.*, 2011; White *et al.*, 2001), consistent with channel phosphorylation by PKA (Quinn *et al.*, 2004; Shi *et al.*, 2007). PKC was also shown to modulate channel activity, for example by inhibition of K^+ current in response to angiotensin II (Kubo *et al.*, 1997; Sampson *et al.*, 2007).

1.3 Vascular endothelium: contrating and relaxing factors

The discovery of endothelium-derived relaxing factor (EDRF) around thirty years ago helped to establish a central role for the endothelium in the physiology and pathophysiology of the cardiovascular system (Furchgott *et al.*, 1980). Now it is well recognised that the endothelium is not just a passive barrier between circulating blood and the vessel wall, but plays a crucial role in inflammation, thrombosis and regulation of local blood flow and systemic blood pressure (Dora, 2010; Feletou *et al.*, 2010b; Garland *et al.*, 2010a).

The vascular endothelium lining the intima of the blood vessels is long known to be a semi-permeable barrier that regulates the conduction of liquid and solutes, including plasma proteins, between the blood and surrounding tissue. Transport across the endothelium occurs via two different pathways: through the endothelial cell (transcellular) or between adjacent cells, through interendothelial junctions (paracellular) (Mehta *et al.*, 2006). Thus, the endothelium creates an interactive diffusion barrier which separates vasoactive substances dissolved in blood plasma from the vascular smooth muscle cells (Lew *et al.*, 1989).

More importantly, the endothelium is also able to regulate tone of adjacent smooth muscle cells directly, by propagation of the electrical signal via gap junctions (de Wit *et al.*, 2010; Dora, 2010; Figueroa *et al.*, 2009a) and by release of vasodilators as well as

vasoconstrictors (Iwatani *et al.*, 2008) in response to different physical and chemical factors, such as shear stress (Katada *et al.*, 2002), hypoxia (Aley *et al.*, 2005), and numerous vasoactive substances. The most important endothelium-derived vasoactive factors are endothelium-derived relaxing, contracting, and hyperpolarizing factors (EDRF, EDCF, and EDHF, respectively).

1.3.1 Endothelial cell Ca^{2+}

Activation of the vascular endothelium is usually associated with a rise in endothelial cell $[Ca^{2+}]_i$, which is released from intracellular Ca^{2+} stores and/or enters from the extracellular space.

It is well known that stimulation of $G_{q/11}$ protein-coupled receptors in rat mesenteric arteries elevates endothelial cell $[Ca^{2+}]_i$ (McSherry *et al.*, 2005; Mumtaz *et al.*, 2011; Oishi *et al.*, 2001; Rodriguez-Rodriguez *et al.*, 2009). The signalling cascade involves phospholipase C-mediated production of IP_3, which evokes efflux of Ca^{2+} from intracellular stores in a similar way as it does in smooth muscle cells (see Section 1.2.2 for details) (Fukao *et al.*, 1997; Mumtaz *et al.*, 2011). Although ryanodine receptors were shown to participate in the $[Ca^{2+}]_i$ rise in human umbilical vein endothelial cells (HUVEC) (Paltauf-Doburzynska *et al.*, 2000), spontaneous $[Ca^{2+}]_i$ events (Kansui *et al.*, 2008) as well as a $[Ca^{2+}]_i$ rise in response to muscarinic receptor stimulation (Mumtaz *et al.*, 2011) were insensitive to ryanodine in endothelial cells from rat mesenteric arteries.

The importance of Ca^{2+} influx from the extracellular space in mesenteric arteries is demonstrated by a decrease in the magnitude of endothelium-dependent hyperpolarization (Dora *et al.*, 2008; Fukao *et al.*, 1997) as well as suppression of $[Ca^{2+}]_i$ responses in low $[Ca^{2+}]_o$(McSherry *et al.*, 2005; Mumtaz *et al.*, 2011). Additionally, attenuation of mesenteric artery hyperpolarization was observed after inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Fukao *et al.*, 1997).

Therefore it was hypothesized that store depletion in endothelial cells activates capacitative Ca^{2+} entry, possibly, via a channel from the TRP family (Mumtaz *et al.*, 2011).

TRP channels are non-selective cation channels that serve as a potent source of Ca^{2+} in many cell types. Endothelial cells were reported to express all, canonical, vanilloid, and melastatin (TRPM) subfamilies of TRP channels (Hill *et al.*, 2006; Yao *et al.*, 2005; Zholos *et al.*, 2011), with TRPC being the main candidate for SOCE (Tiruppathi *et al.*, 2006; Yao *et al.*, 2005). In accordance to this, it was shown that the TRPC channel may form a complex with IP_3 receptors in order to optimize Ca^{2+} signalling (Rath *et al.*, 2009), although discrepancies between SOCE and TRPC signalling profiles may argue against TRPC being solely responsible for the capacitative Ca^{2+} entry (Nilius, 2004).

Another important pathway of Ca^{2+} entry may be via myo-endothelial gap junctions. It was shown for rat mesenteric arteries that stimulation of smooth muscle cells with a Ca^{2+} raising agent may result in increased $[Ca^{2+}]_i$ in adjacent endothelial cell (Dora *et al.*, 2000; Oishi *et al.*, 2001). This may serve as a negative feedback aimed to reduce vascular tone in response to excessive elevation of smooth muscle cell $[Ca^{2+}]_i$ through the release of dilator factors or hyperpolarization.

Lastly, endothelial cell cyclic nucleotide gated channels may play a role in endothelium-dependent vascular dilation to a number of cAMP-elevating agents including adenosine, adrenaline (Shen *et al.*, 2008) and ATP (Kwan *et al.*, 2010).

Regardless of the source, increases in $[Ca^{2+}]_i$ will potentially stimulate endothelial nitric oxide synthase (eNOS) and cyclooxygenase (COX) enzymes, leading to the release of nitric oxide (NO) and prostanoids, respectively. It can also stimulate the intermediate and small conductance Ca^{2+}-sensitive K^+ channels (IK_{Ca} and SK_{Ca},

respectively); activation of these channels will cause efflux of K^+ and hyperpolarization of the endothelium (Chen *et al.*, 1997; Edwards *et al.*, 1998; Zygmunt *et al.*, 1996). Therefore, endothelium-dependent modulation of vascular tone can occur via Ca^{2+}-dependent formation of NO, prostanoids, and hyperpolarization.

1.3.2 Nitric oxide signalling pathway

Shortly after the discovery of the obligatory role for the endothelium in dilatation response to acetylcholine (ACh) (Furchgott *et al.*, 1980), the close similarities between EDRF and nitric oxide (NO) were noticed (Ignarro *et al.*, 1987; Palmer *et al.*, 1987), establishing a whole new era in vascular research (Fleming, 2010).

It was found that in the vascular endothelium NO is generated by endothelial nitric oxide synthase (eNOS), an enzyme which converts L-arginine to L-citrulline and NO (Bredt *et al.*, 1990; Palmer *et al.*, 1988). The functional enzyme is a dimer, with each monomer consisting of an oxygenase domain that includes a binding site for heme, tetrahydrobiopterin (BH_4) and L-arginine, and a reductase domain that contains binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavines and calmodulin. Upon activation, electrons migrate from NADPH through flavines, BH_4 and calmodulin to the heme, which binds O_2 and catalyses NO production from L-arginine (Fleming, 2010). In order to be activated, eNOS, with help of dynamin, has to be translocated from the plasma membrane to the Golgi apparatus, where it can dissociate from caveolin-1 and bind calmodulin (Cao *et al.*, 2001; Figueroa *et al.*, 2002; Fleming, 2010; Maniatis *et al.*, 2006). Although this is believed to be the predominant mechanism of endogenic NO synthesis, several other pathways of possible NO production have been suggested, such as non-enzymatic NO release via reaction of hydrogen peroxide (H_2O_2) and arginine (Nagase *et al.*, 1997), or during acidic conditions via reduction of inorganic nitrite (Modin *et al.*, 2001).

There are diverse stimuli that can lead to eNOS activation in rat mesenteric arteries. This can be signalling of endothelial G protein-coupled receptor (Simonsen *et al.*, 1999; Wu *et al.*, 1997), shear stress (Koller *et al.*, 1994; Liu *et al.*, 2006), or even Ca^{2+} incoming from smooth muscle cells via myo-endothelial gap junctions (Dora *et al.*, 1997; Dora *et al.*, 2000). Also, NO is basally produced by rat arterial endothelium in resting conditions (Figueroa *et al.*, 2009b; Fukuda *et al.*, 1992).

Initially, eNOS was classified as a Ca^{2+}-calmodulin-dependent enzyme and its activation upon muscarinic receptor stimulation was attributed predominantly to elevation of $[Ca^{2+}]_i$ (Bredt *et al.*, 1990). However, recent investigations have revealed that phosphorylation of the enzyme may enhance or reduce the sensitivity to $[Ca^{2+}]_i$, thus enabling NO production at resting levels of Ca^{2+} or suppressing its signalling even if $[Ca^{2+}]_i$ is elevated (Fleming, 2010). eNOS activity was shown to be altered by phosphorylation or dephosphorylation of stimulatory (Ser 635 and Ser 1177) or inhibitory (Ser116 and Thr 495) sites by a wide range of kinases and phosphatases, such as serine/threonine (Akt) kinase, Rho kinase, protein phosphatase 2A and PKC (Dudzinski *et al.*, 2006; Hirata *et al.*, 1995; Matsuda *et al.*, 2006; Seya *et al.*, 2006). eNOS is also highly dependent on calmodulin, which by itself is under the control of protein casein kinase 2 (CK2) (Greif *et al.*, 2004). In cultured cells, PKA was shown to have no effect (Hirata *et al.*, 1995) or a stimulatory effect on eNOS activity (Hashimoto *et al.*, 2006). In addition, PKA upregulates PKC and CK2 (Blanquet, 1998; Wooten *et al.*, 1996), which each have an inhibitory influence on eNOS (Greif *et al.*, 2004; Hirata *et al.*, 1995). Interestingly, NO production in the endothelium from resistance arteries can also be triggered by the β-subunit of G_q or $G_{i/o}$ proteins (Vequaud *et al.*, 2001).

Although the uncharged free radical state of the NO molecule ($NO^{\bullet}$) was thought to be the main EDRF (Dierks *et al.*, 1996b; Feelisch *et al.*, 1994; Ignarro *et al.*, 1987),

nitric oxide can also exist as nitrosonium cation (NO^+) and nitroxyl anion (NO^-) (Hughes, 1999). It was demonstrated that NO^- can also potently dilate rat aorta (Pino *et al.*, 1994b) or mesenteric arteries (Irvine *et al.*, 2003a; Wanstall *et al.*, 2001); moreover, scavenging NO^- with L-cysteine has uncovered the endogenous production of this molecule by vascular tissues (Andrews *et al.*, 2009; Ellis *et al.*, 2000; Rajanayagam *et al.*, 1993; Wanstall *et al.*, 2001; Yuill *et al.*, 2010).

Arteries dilate to NO by means of at least three different mechanisms. The first mechanism includes smooth muscle cell hyperpolarization, which has been shown to be a result of opening of K_{ATP} (Garland *et al.*, 1992a) or K_{Ca} channels (Archer *et al.*, 1994; Mistry *et al.*, 1998; Sampson *et al.*, 2001; White *et al.*, 1993; Yuill *et al.*, 2010). Recently, NO^- was also shown to hyperpolarize smooth muscle cells via activation of K_v channels (Andrews *et al.*, 2009; Yuill *et al.*, 2010). Although there is evidence of a direct action of NO on K^+ channels (Bolotina *et al.*, 1994; Mistry *et al.*, 1998), the main pathway involves activation of guanylyl cyclase via binding of NO with the prosthetic heme of the enzyme. This leads to formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), which promotes translocation of soluble protein kinase G (PKG) to the plasma membrane (Carvajal *et al.*, 2000).

PKG is also responsible for a second and third pathway of NO-mediated dilatation, which includes reduction of smooth muscle Ca^{2+} and desensitization of the contractile apparatus to Ca^{2+}. PKG reduces smooth muscle cell $[Ca^{2+}]_i$ by means of direct suppression of the L_{Ca} channel (Liu *et al.*, 1997; Sumii *et al.*, 1995), inactivation of IP_3 receptors (Komalavilas *et al.*, 1996), and stimulation of plasma membrane Ca^{2+}-ATPase (Furukawa *et al.*, 1988) and SERCA (Andriantsitohaina *et al.*, 1995). And, lastly, PKG increases MLC phosphatase activity (Etter *et al.*, 2001; Iwatani *et al.*, 2007; Somlyo *et al.*, 2003), contributing to a reduction in vascular tone.

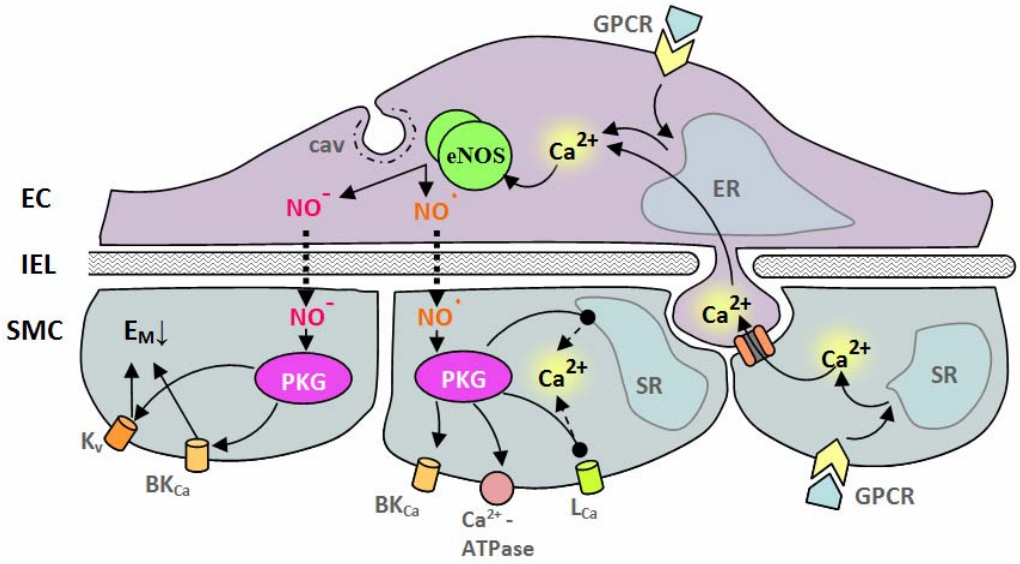


Figure 1.1 Diagram illustrating the signalling of eNOS in resistance arteries

A rise in endothelial cell (EC) $[Ca^{2+}]_i$ in response to stimulation of $G_{q/11}$–coupled receptors or due to a Ca^{2+} signal from an adjacent smooth muscle cell simulates eNOS leading to the production of $NO^{\bullet}$ and NO^{-}, which diffuse to underlying smooth muscle cells (SMC) where they trigger production of cGMP by guanylyl cyclase. $NO^{\bullet}$ can act directly (not shown) or via PKG, suppressing Ca^{2+} release from intracellular stores, stimulating Ca^{2+} extrusion by the Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase and Na^{+}/Ca^{2+} exchanger, and Ca^{2+} uptake via SERCA. $NO^{\bullet}$ signalling can also stimulate BK_{Ca} and K_{ATP} channels, and suppresses L_{Ca} channels. NO^{-} acts mainly via PKG, promoting opening of K_v and BK_{Ca} channels.

1.3.3 Endothelium-derived hyperpolarizing factor

The ability of the endothelium from resistance arteries to evoke smooth muscle hyperpolarization emerged soon after the discovery of EDRF (Bolton *et al.*, 1984). It was found later that this hyperpolarization was not mediated by NO or prostacyclin (Chen *et al.*, 1988; Garland *et al.*, 1992a; Plane *et al.*, 1996), but was thought to be evoked by an unidentified factor, EDHF (Taylor *et al.*, 1988; Waldron *et al.*, 1994). Nowadays it is clear that the hyperpolarization relies not on single pathway, but several complex pathways, therefore it can be more accurately described as endothelium-derived hyperpolarization (EDH) (Dora, 2010; Garland *et al.*, 2010a). At least three pathways of EDH have been identified in resistance arteries: K^+ released by the endothelium, hyperpolarization transferred via myo-endothelial gap junctions, and diffusible hyperpolarizing factors.

K^+ as EDH

The major role for EDH in small arteries is based, at least in part, on the enhanced dependency of contraction of these vessels on voltage-gated Ca^{2+} channels, and, therefore, the bigger reliance of vascular tone on smooth muscle membrane potential (for review please see Garland *et al.*, 2010a; Taylor *et al.*, 1988). The membrane potential is largely controlled by K^+ channels, and the idea that K^+ channels predominantly contribute to the EDH was supported by the evidence that ACh evokes ^{86}Rb efflux form the ^{86}Rb-treated arteries (Chen *et al.*, 1988). Apparently, neither K_{ATP} channels (Garland *et al.*, 1992b) nor K_v channels (Adeagbo *et al.*, 1993) were responsible for the hyperpolarization, but inhibition of SK_{Ca} and IK_{Ca} channels (Adeagbo *et al.*, 1993; Doughty *et al.*, 1999; Zygmunt *et al.*, 1996) as well as K_{ir} channels and Na^+/K^+-ATPase (Edwards *et al.*, 1998) suppressed EDH. Therefore it was hypothesized that efflux of K^+ from endothelial SK_{Ca} and IK_{Ca} channels may activate

smooth muscle K_{ir} and Na^+/K^+-ATPase and thus cause smooth muscle cell hyperpolarization. This hypothesis was proven to be true by means of a K^+-selective electrode that measured a 10 mM increase in K^+ close to myo-endothelial space after stimulation of the artery with ACh, and by rise of extracellular K^+ mimicking the endogenous EDH (Edwards *et al.*, 1998). This pathway, however, can be masked with a high level of tone via saturation of Na^+/K^+-ATPase with K^+ incoming from BK_{Ca} channels (Dora *et al.*, 2002), or when levels of Ca^{2+} in the myo-endothelial space increase, for example, when smooth muscle L_{Ca} are inactive (Dora *et al.*, 2008). Under these circumstances EDH may rely solely on a second pathway, namely direct transfer of hyperpolarization via myo-endothelial gap junctions.

Direct transfer of hyperpolarization via myo-endothelial gap junctions

It was clearly demonstrated that whilst IK_{Ca} channels are co-expressed together with Na^+/K^+-ATPase within the myo-endothelial projections, SK_{Ca} channels are also localized at endothelial cell borders (Dora *et al.*, 2008) in caveolin-rich domains, together with TRP channels and eNOS (Rath *et al.*, 2009). This difference in localization may underlie the difference in EDH pathway that includes these channels signalling.

It was established that hyperpolarization to ACh in endothelial cells is similar to that in adjacent smooth muscle cells (Yamamoto *et al.*, 1999). Therefore, it was proposed that myo-endothelial gap junctions may provide a pathway for EDH being transferred from the endothelium to the smooth muscle (Griffith *et al.*, 2002). Immunological studies revealed the presence of these structures in the area of myo-endothelial projections, and that their incidence increased with decrease in arterial size (Sandow *et al.*, 2000). Indeed, suppression of gap junctional communication affected the degree of hyperpolarization and EDH-dependent dilatation in resistance arteries

(Dora *et al.*, 1999; Dora *et al.*, 2003a). Inhibition of connexin-40 with the specific anti-conexin antibody also suppressed the EDH pathway; the inhibition was especially evident when a high level of tone was used in order to affect signalling via the Na^+/K^+-ATPase-dependent mechanism described above (Mather *et al.*, 2005). When gap junctions were blocked, inhibition of the IK_{Ca} channel or Na^+/K^+-ATPase alone was sufficient to inhibit the EDH in rat mesenteric arteries, indicating that SK_{Ca} channels are responsible for the hyperpolarization that transfers from the endothelium via gap junctions, whilst IK_{Ca} channels most probably provide K^+ for Na^+/K^+-ATPase-mediated repolarization of the smooth muscle cell (Dora *et al.*, 2008). Co-expression of SK_{Ca} channels with K_{ir} channels at endothelial cell borders (Dora *et al.*, 2008) may provide an amplification mechanism, impairment of which underlies the reduced EDH response in the hypertensive rat model (Weston *et al.*, 2010).

Diffusible hyperpolarizing factors

Although in rat mesenteric arteries the above pathways are predominant routes for EDH, we cannot rule out the possibility that other diffusible hyperpolarizing factors may also play a role. It is known, for example, that increases in endothelial cell $[Ca^{2+}]_i$ also activates phospholipase A leading to production of arachidonic acid, a substrate for epoxyeicosatrienoic acids (EETs) formation by cytochrome P-450. EETs were shown to evoke hyperpolarization via K_{Ca} channels (Campbell *et al.*, 1996), possibly via stimulation of TRP channels (Campbell *et al.*, 2010) or ryanodine receptors closely located to the channel (Earley *et al.*, 2005). Athough EETs were also shown to be selective TP receptors antagonists (Fetalvero *et al.*, 2007), which can explain relaxation produced by EETs apart from participation in EDHF signalling.

Following administration of carbachol, the endothelium was shown to produce another product of cytochrome P-450, cannabinoid anandamide (Mechoulam *et al.*,

1998), which hyperpolarized mesenteric arteries in a similar manner as EDH (Randall *et al.*, 1997). However, application of anandamide inhibited the endogenous EDH response in porcine coronary arteries (Fleming *et al.*, 1999b).

C-natriuretic peptide was also proposed as candidate for EDHF. In support of this, it was demonstrated that C-natriuretic peptide can be released by the endothelium (Chauhan *et al.*, 2003; Hobbs *et al.*, 2004); moreover, the profile of hyperpolarization evoked by C-natriuretic peptide, e.g., sensitivity to ouabain and Ba^{2+}, were similar to those evoked by endogenous EDH in rat mesenteric arteries (Chauhan *et al.*, 2003). On the other hand, differences in EDH and responses to C-natriuretic peptide in guinea-pig coronary artery (Leuranguer *et al.*, 2008) discounted a possible role for C-natriuretic peptide as EDHF (Garland *et al.*, 2008).

Another putative EDHF may be H_2O_2 (Matoba *et al.*, 2003; Shimokawa *et al.*, 2004), which can be produced by endothelial eNOS (Fleming, 2010), COX (Sobey *et al.*, 1998), and NADPH oxidase (Zhou *et al.*, 2008) in sufficient amounts to cause hyperpolarization (Beny *et al.*, 1991). But H_2O_2 can also cause contraction (Gao *et al.*, 2001; Lucchesi *et al.*, 2005) as well as be responsible for endothelial dysfunction in hypertensive rat models (Gao *et al.*, 2001; Zhou *et al.*, 2008), which makes it unlikely to be the EDHF.

Regardless the mechanism, all three pathways of hyperpolarization will lead to closure of L_{Ca} channels, thus reduce smooth muscle cell $[Ca^{2+}]_i$ and, therefore, cause vasodilatation.

1.3.4 Endothelium-derived contracting factor

The first endothelium-derived factor discovered was prostacyclin (PGI_2) (Moncada *et al.*, 1976), a product of COX metabolism of arachidonic acid that potently dilates conduit arteries via activation of the cAMP pathway in smooth muscle cells (Kukovetz *et al.*, 1979; Miller *et al.*, 1979); however, it was soon noticed that PGI_2 can evoke not only vasodilatation, but also vasoconstriction (Dusting *et al.*, 1977). Other COX metabolites, such as prostaglandin (PG)H_2, PGE_2, PGD_2, $PGF_{2\alpha}$, and thromboxane (Tx)A_2, exhibit even higher vasoconstrictor properties, in part due to their ability to bind to thromboxane prostanoid (TP) receptor (Feletou *et al.*, 2010a). In physiological conditions, COX activity is mainly controlled by $[Ca^{2+}]_i$ directly (Tang *et al.*, 2007) or via enhanced production of arachidonic acid (Wong *et al.*, 2010a); kinases, such as PKA, were also shown to modulate its function (Samokovlisky *et al.*, 1999).

Although prostanoids are the main contributors of EDCF (Feletou *et al.*, 2010b), one of the most potent vasoconstrictors known is an endothelium-derived 21-amino acid peptide, endothelin-1 (Yanagisawa *et al.*, 1988). In the vasculature, endothelins mediate their action via two G-protein-coupled receptor subtypes, ET_A and ET_B, with ET_A being expressed predominantly by the vascular smooth muscle cells and being mainly responsible for the vasoconstriction to endothelin-1 (Gilbert *et al.*, 2001; Maguire *et al.*, 1994; Montagnani *et al.*, 2000).

The vascular endothelium, especially in pathophysiological conditions, produces large amounts of reactive oxygen species (ROS) (Tang *et al.*, 2007; Zhou *et al.*, 2008). ROS can evoke vasoconstriction by Rho-kinase-mediated phosphorylation of MLC phosphatase (Knock *et al.*, 2009) or via enhancement of TP receptor signalling (Gao *et al.*, 2004). ROS can also facilitate eNOS uncoupling, which causes further ROS formation (Dikalova *et al.*, 2010; Gayen *et al.*, 2010).

1.4 Propagated vasomotor responses

One of the important characteristics of resistance arteries is the ability to synchronise activity within a certain length of the vessel segment. It was noticed that hyperpolarization originating in endothelial cells can spread radially into the adjacent smooth muscle cells (Emerson *et al.*, 2001; Emerson *et al.*, 2000; Takano *et al.*, 2004; Yamamoto *et al.*, 1999; Yamamoto *et al.*, 2001), whilst changes in the membrane potential of smooth muscle can cause similar membrane potential changes in the endothelium of some blood vessels (Marchenko *et al.*, 1994; Muraki *et al.*, 2000). Intercellular connections in resistance arteries were also demonstrated by microinjection of a fluorescent dye that could readily spread from the injected endothelial cell to a neighbouring endothelial cell via gap junctions and, less effectively, to adjacent smooth muscle cells (Dora *et al.*, 2003a; Takano *et al.*, 2004). Dye spread between smooth muscle cells was shown to be limited (Takano *et al.*, 2004), especially in hypertensive animals (Sandow *et al.*, 2003). These experiments suggest a presence of both homocellular and heterocellular gap junctions; this assumption is supported by electron microscopy studies showing prevalant interendothelial gap junctions and smaller clusters of myo-endothelial gap junctions in rat resistance arteries (Sandow *et al.*, 2000).

Generally, gap junctions are formed by the docking of two connexons (hemichannels) from opposing cells. Each connexon contains six protein subunits, called connexins, that can change the conformation of their extracellular loops in order to form an aqueous pore that allows diffusion of charged molecules up to 1 kDa between the connected cells (de Wit *et al.*, 2010; Figueroa *et al.*, 2009a). Four connexin subtypes were found in the vasculature: Cx37, Cx40, Cx43 and Cx45, with the first three subtypes playing the predominant role in rat mesenteric arteries (Kansui *et al.*, 2004; Sandow *et al.*, 2006). Markedly, whilst IK_{Ca}, Cx40 and Cx37 were co-expressed

in the myo-endothelial gap junction-associated endothelial cell projection, SK_{Ca}, Cx37, Cx40 and Cx43 were localized to adjacent endothelial cell gap junctions, indicating differential signalling pathways of this microdomains (Sandow *et al.*, 2009; Sandow *et al.*, 2006). Despite the expression of all three connexins, inhibition of Cx40 alone could block the EDH response in rat mesenteric arteries (Mather *et al.*, 2005). A role of gap junctions in the coordination of smooth muscle and endothelial membrane potential is also confirmed by the evidence that in the vessels lacking myo-endothelial gap junctions, such as femoral or saphenous arteries of adult animals, conductance of hyperpolarizing currents is greatly reduced (Sandow *et al.*, 2004; Sandow *et al.*, 2002).

Gap junctional permeability can be regulated by a range of mechanisms, such as phosphorylation of connexins by PKC (Heyman *et al.*, 2009) or PKA (Griffith *et al.*, 2002; Popp *et al.*, 2002; van Rijen *et al.*, 2000), Epac-mediated Cx43 phosphorylation (Duquesnes *et al.*, 2010) and changes in $[Ca^{2+}]_i$ (Matchkov, 2010), enabling a fine control of the propagated vasomotor response both radially and longitudinally.

1.4.1 Conducted vasodilatation

In order to achieve a significant increase in tissue blood flow, it is important that the focal dilatation to a vasodilator agent could evoke response that spreads axially along the vessel wall and, therefore, enables a wide drop in vascular resistance. Homocellular and heterocellular gap junctions provide a pathway for such spreading, or 'conducted', dilatation (de Wit *et al.*, 2010; Dora, 2010; Garland *et al.*, 2010a).

Initially, the conducted response was thought to be a consequence of a simple current transfer to adjacent cells (Segal *et al.*, 1989). Indeed, vasodilators that do not lead to potent changes in membrane potential, such as $NO^{\bullet}$ donors, were shown to evoke poor or no conducted dilatation (Delashaw *et al.*, 1991; Dora, 2010; Winter *et al.*, 2007), whilst agonists which can cause potent hyperpolarization evoked dilatation that

was able to spread over 2 mm from the site of application (Dora *et al.*, 2003b; Garland *et al.*, 2011; Segal *et al.*, 1999; Takano *et al.*, 2004; Winter *et al.*, 2007; Yuill *et al.*, 2010). However, the fact that hyperpolarization evoked by ACh decayed less than the injected current demonstrates the presence of an as yet unidentified amplification mechanism (Emerson *et al.*, 2002). A role for K_{ir} channels has been suggested (Goto *et al.*, 2004; Jantzi *et al.*, 2006), but experiments performed in rat mesenteric arteries have shown no effect of K_{ir} channels inhibition on the magnitude of the conducted dilatation (Takano *et al.*, 2004). Among other candidates, Na^+/K^+-ATPase and/or NCX are present (Dora, 2010; Garland *et al.*, 2010a; Matchkov, 2010). Voltage-dependent Na^+ channels via activation of Ca^{2+} influx through endothelial L_{Ca} channel were also shown to contribute to conducted dilatation in mouse cremaster arteries (Figueroa *et al.*, 2007). Although a rise in $[Ca^{2+}]_i$ seems unlikely to be the amplification mechanism because, on the one hand, hyperpolarization was shown not to alter endothelial cell $[Ca^{2+}]_i$ (McSherry *et al.*, 2005), and, on the other hand, no changes in $[Ca^{2+}]_i$ were observed during conducted responses in rat mesenteric arteries (Takano *et al.*, 2004). However, this possibility cannot be completely excluded since a slow $[Ca^{2+}]_i$ wave was detected in endothelium of skeletal muscle arterioles (Tallini *et al.*, 2007; Uhrenholt *et al.*, 2007).

Since endothelium-independent agonists, such as levcromakalim, isoprenaline or NO^- could evoke conducted dilatation (Garland *et al.*, 2011; Takano *et al.*, 2004; Yuill *et al.*, 2010), it may seem that the endothelium does not play a significant role in this process; however, endothelium removal prevented spread of the dilatation in response to all tested agonists (Garland *et al.*, 2011; Takano *et al.*, 2004; Yuill *et al.*, 2010). This may be explained in two ways. First, endothelial cells possess a much higher dencity of gap junctions than smooth muscle cells (Sandow *et al.*, 2003; Sandow *et al.*, 2000), thus, providing a better intercellular communication pathway, and second, endothelial

cells align along the principal axis of the artery, therefore less intercellular connections are needed in order to conduct the signal for longer distances (Garland *et al.*, 2010a).

1.4.1 Synchronization of vasomotion

Vasomotion is seen as oscillations in tension or diameter associated with corresponding oscillations in membrane potential and $[Ca^{2+}]_i$ that are spatially uniform within individual smooth muscle cells and synchronous between adjacent smooth muscle cells (Gustafsson, 1993; Peng *et al.*, 2001).

The endothelium clearly plays a leading role in the coordination of vasomotion in rat mesenteric arteries since endothelial denudation resulted in loss of coordinated oscillations of vascular tone in this tissue (Gustafsson, 1993; Mauban *et al.*, 2004; Peng *et al.*, 2001; Rahman *et al.*, 2007). Participation of the eNOS/cGMP system in the generation and maintenance of these rhythmical tone oscillations was initially hypothesised and the synchronising effect of a membrane-permeable analogue of cGMP on vasomotion was clearly shown in rat resistance arteries (Gustafsson, 1993; Peng *et al.*, 2001; Rahman *et al.*, 2005); however, further studies revealed the desynchronising effect of endothelium-derived NO (Sell *et al.*, 2002; Yuill *et al.*, 2009) and even supported a leading role of EDHF in initiating and maintaining vasomotion (Mauban *et al.*, 2004; Rahman *et al.*, 2007). Vasomotion was abolished when the activity of chloride channels, L_{Ca} channels and/or the Na^+/K^+-ATPase was blocked (Boedtkjer *et al.*, 2008; Gustafsson, 1993). The current model suggests that initial asynchronous oscillations in Ca^{2+} in individual cells activates a depolarizing current, which can spread via homocellular and myoendothelial gap junctions and after achieving a certain threshold, evoke simultaneous depolarization of all smooth muscle cells, which will cause activation of L_{Ca} channels, simultaneous $[Ca^{2+}]_i$ elevation and vasoconstriction, thus initiate synchronous vasomotion (Peng *et al.*, 2001; Rahman *et al.*, 2007).

1.5 Receptors regulating vascular tone

Since blood carried by arteries plays such a crucial role in tissue homeostasis, there are multiple pathways for controlling the level of blood flow to tissues and organs. In the majority of cases, this control is enabled by means of numerous receptors expressed in both smooth muscle and endothelial cells. Interaction of these receptors with a variety of vasoactive molecules released by perivascular nerve endings (Kawamura *et al.*, 1989; Lamont *et al.*, 2003) or present in circulating blood through a complex network of signal transduction pathways act to modulate the level of vascular tone and, subsequently, the blood flow. Adrenergic, muscarinic, and TP receptors are the most widely expressed receptors in vascular tissue, and therefore, their downstream signaling significantly contributes to the regulation of the circulation.

1.5.1 Vascular adrenoceptors

Adrenergic receptors (adrenoceptors) are activated by binding of endogenous catecholamines adrenaline and noradrenaline (NA). NA is synthesized in noradrenergic neurons and the adrenal medulla from the amino acid L-tyrosine. Methylation of NA by phenylethanolamine N-methyltransferase to produce adrenaline occurs only in chromaffin cells of adrenal medulla. Vesicles containing adrenaline or NA are discharged upon depolarization (Marcantoni *et al.*, 2007). Concentrations of adrenaline and NA in rat plasma reach 3 ng/ml and 1.6 ng/ml respectively, and this amount increases several fold during exercise and disease (Barbieri *et al.*, 1996; Benthem *et al.*, 1995; De Boer *et al.*, 1987). The action of NA/adrenaline is terminated mainly by their reuptake into the nerve endings or chromaffin cells, a process controlled by presynaptic adrenoceptors, where they can be recycled or degraded (Dunn *et al.*, 1999; Floras, 1992).

Adrenoceptors are seven-transmembrane domain G protein-coupled receptors, with nine different subtypes currently being cloned and characterized (for review see Guimaraes *et al.*, 2001).

α_1-adrenoceptors are coupled to G_q proteins, which stimulate phospholipase C and through elevation of $[Ca^{2+}]_i$ cause vasoconstriction (see Section 1.2.2 for details). α_1-adrenoceptors can be further subdivided into α_{1A}, α_{1B}, and α_{1D} subtypes; a postulated fourth α_1-adrenoceptor subtype, the α_{1L}-adrenoceptor, seems to represent a functional phenotype of the α_{1A}-adrenoceptor in a particular, energetically favourable, conformational state (Docherty, 2010; Guimaraes *et al.*, 2001). Contractile responses of the smooth muscle of rat mesenteric arteries to NA/adrenaline were shown to be predominantly mediated via α_{1D}-adrenoceptors, with α_{1B}-adrenoceptors found to be only partly involved (Hussain *et al.*, 2000; Piascik *et al.*, 1997); however, immunohistochemical and mRNA analysis demonstrated expression of all three cloned α_1-adrenoceptor subtypes in this tissue (Hrometz *et al.*, 1999; Xu *et al.*, 1997). A functional response of endothelial cells to stimulation of α_{1D}-adrenoceptors was also shown in the mesenteric vascular bed (Filippi *et al.*, 2001), although this effect may be mediated by an indirect influence of smooth muscle α_1-adrenoceptor signaling (Dora *et al.*, 1997; Dora *et al.*, 2000).

α_2-adrenoceptors are linked to G_i proteins which inhibit adenylyl cyclase and in vascular tissue have three subtypes: $\alpha_{2A/D}$, α_{2B}, and α_{2C} (Guimaraes *et al.*, 2001). Receptor activation can also cause vascular smooth muscle contraction, particularly in veins (Paiva *et al.*, 1999). In rat mesenteric arteries, a selective α_2-adrenoceptor agonist, clonidine, was shown to evoke both depolarization/contraction and hyperpolarization/relaxation, where depolarization was an effect of partial activation of

α_1-adrenoceptors, and hyperpolarization was a result of via α_2-adrenoceptor-mediated opening of K^+ channels (Silva *et al.*, 1996).

Whilst evidence of α_2-adrenoceptor functional relevance in mesenteric artery smooth muscle cell is poor, clonidine caused concentration-dependent dilatation of the artery via NO production by the endothelium (Bockman *et al.*, 1996; Borhani *et al.*, 2005; Figueroa *et al.*, 2001). The main subtype participating in the dilatation was shown to be $\alpha_{2A/D}$, which, surprisingly, did not mediate its effect via cAMP (Bockman *et al.*, 1996; Figueroa *et al.*, 2001). Possibly, the β subunit of G_i protein is responsible for the eNOS activation in this case (Vequaud *et al.*, 2001).

There are three main subtypes of β-adrenoreceptor: β_1, β_2 and β_3, all linked to adenylyl cyclase through G_s protein, which leads to vessel dilatation through mechanisms described in Section 1.2.3; although a link between the β_3 subtype and G_i protein has also been reported (Guimaraes *et al.*, 2001).

Distribution of β-adrenoceptor subtypes depends on the vascular bed and species. β_1-adrenoceptor-mediated relaxation of smooth muscles was thought to predominate in coronary and cerebral arteries (Begonha *et al.*, 1995; Edvinsson *et al.*, 1974), whilst the β_2 subtype is widely distributed among the majority of blood vessels. The atypical β_3 subtype, which initially was considered to participate mainly in lipolysis in rat adipocytes (Harms *et al.*, 1974), later was suggested to play a significant role in control of vascular tone (Dessy *et al.*, 2004; MacDonald *et al.*, 1999; Shen *et al.*, 1994; Sooch *et al.*, 1995), particularly, when resistance of β_3 adrenoceptor to desensitization was taken into account (Liggett *et al.*, 1993). However, more extensive pharmacological studies revealed that the atypical β-adrenoceptor in the vasculature was distinct from the β_3-adrenoceptor and was suggested to be a "putative β_4-adrenoceptor" (Brawley *et al.*, 2000; Kozlowska *et al.*, 2003; Torrens *et al.*, 2002), although parallel studies revealed

that this atypical β-adrenoceptor differed from the putative β_4-adrenoceptor too, and was just a low-affinity state of the β_1-adrenoceptor (Kaumann *et al.*, 2001). A recent study by our group confirmed an earlier observation that in the dilatation of rat mesenteric arteries to β-adrenergic agonists β_1-adrenoceptors are predominantly involved, and β_2-adrenoceptors play only a secondary role (Briones *et al.*, 2005; Garland *et al.*, 2011; Zwaveling *et al.*, 1996).

Another controversy exists regarding the contribution of endothelial β-adrenoceptors in the regulation of vascular tone (see Section 4.1 for details). The main role seems to belong to β_3 adrenoceptor-mediated NO production (Dessy *et al.*, 2004; Dessy *et al.*, 2005; Figueroa *et al.*, 2009b; Kou *et al.*, 2007; Napp *et al.*, 2009); however, this receptor was shown not to participate in the responses to β-adrenergic agonists in rat mesenteric arteries (Garland *et al.*, 2011).

1.5.2 Vascular muscarinic cholinergic receptors

Muscarinic cholinoceptors are seven-transmembrane domain receptors coupled to $G_{q/11}$ protein. They are activated by a cholinergic neurotransmitter ACh, synthesized by choline acetyltransferase from choline and acetyl-CoA, with the concentration reaching ~1.2 nM in rat blood plasma (Fujii *et al.*, 1995). Signalling of all five subtypes of the receptor causes rise in $[Ca^{2+}]_i$ via phospholipase C-mediated PIP_2 breakdown (Eglen, 2006) (see Section 1.2.2 for details).

ACh mainly activates endothelial muscarinic M_3 receptors in rat mesenteric arteries (Fujimoto *et al.*, 1991; Wu *et al.*, 1997), which was shown to be localized at endothelial cell borders near gap junctions as well as endothelial cell projections (Rodriguez-Rodriguez *et al.*, 2009). Activation of the receptor is associated with a rise in endothelial $[Ca^{2+}]_i$ (McSherry *et al.*, 2005; Oishi *et al.*, 2001; Rodriguez-Rodriguez *et*

al., 2009), which leads to formation of EDRF, EDCF and EDH via mechanisms described earlier.

1.5.2 Thromboxane receptor

The thromboxane (TP) receptor is a seven-transmembrane domain receptor that is coupled to G_q and $G_{12/13}$ proteins leading to activation of phospholipase C and a small GTPase RhoA (Hirata *et al.*, 1996; McKenzie *et al.*, 2009; Zhang *et al.*, 2006a). It is activated by thromboxane A_2, a product of enzymatic cleavage of PGH_2 by thromboxane synthase located mainly in platelets or the endothelium. Blood plasma thromboxane metabolite (thromboxane B_2) concentration in healthy rats is ~7.5 nM, whilst in the hypertensive rat model it reaches ~10.4 nM (Sugimoto *et al.*, 2000). Although thromboxane A_2 is the most potent agonist, other eicosanoids, such as prostacyclin and prostaglandins, are also able to activate the TP receptor. This may lead to potent vasoconstriction and inflammation of the endothelium, contributing to pathophysiological processes in the vasculature (Feletou *et al.*, 2009; Wong *et al.*, 2010b).

So far, two subtypes of the TP receptor have been identified, the first one being characterized for placenta (TP-P(α)) and the second one being expressed in the vascular endothelium (TP-E(β)) (Hirata *et al.*, 1996; Raychowdhury *et al.*, 1994). Both subtypes are coupled to phospholipase C, however, TP-P(α) activates, whilst TP-E(β) inhibits adenylyl cyclase (Hirata *et al.*, 1996). TP receptor activation was also shown to impair cAMP-signalling via Rho kinase-dependent stimulation of phosphodiesterases (Liu *et al.*, 2010).

Immunohistochemical analysis demonstrated that rat mesenteric artery smooth muscle cells express TP receptors (Zhang *et al.*, 2009a), activation of which evokes asynchronous $[Ca^{2+}]_i$ oscillations and potent constriction that is associated with a greater

Ca^{2+}-sensitization than mediated by α-adrenoceptors (Shaw *et al.*, 2004). The endothelium of human arteries (Raychowdhury *et al.*, 1994; Shirasaki *et al.*, 2007) as well as of rabbit aorta (Pfister, 2008) was shown to express TP receptors; although, to our knowledge, TP receptor expression in the endothelium of rat mesenteric arteries has not been yet shown. Endothelial TP receptor activation leads to Rho kinase-dependent inhibition of NO production (Liu *et al.*, 2009), and/or to PKC-mediated uncoupling of eNOS and release of ROS (Zhang *et al.*, 2010). Moreover, TP receptor signalling was shown to prevent insulin-dependent phosphorylation of eNOS at the stimulatory Ser 1177, and this pathway was implicated in vascular dysfunction during high fat diet-induced diabetes in mice (Song *et al.*, 2009).

ROS that can be released by uncoupled eNOS (Zhang *et al.*, 2010) may evoke contraction via TP receptor signalling (Gao *et al.*, 2001), seemingly, acting on COX and promoting thromboxane A_2 production (Garcia-Redondo *et al.*, 2009). Released thromboxane A_2 can bind to endothelial TP receptors, leading to further prostacyclin production (Hunt *et al.*, 1992); prostacyclin in turn can also activate TP receptors, contributing to altered signalling, that may lead to endothelial dysfunction, hypertension and atherosclerosis (Belhassen *et al.*, 2003; Feletou *et al.*, 2009).

1.5 Endothelial dysfunction in age and disease.

Directly or indirectly blood vessels are the source of many serious diseases. Systemic hypertension is a well-known disorder that leads to increased risks of cardiovascular and renal complications and reduced life expectancy. There are several identified reasons for development of high blood pressure, such as tumours of the adrenal medulla and adrenal cortex, which can be treated and therefore lead to restoration of normal blood pressure; however, the causes of essential hypertension, a persistent rise in blood pressure without an identified reason, are not yet clarified (Dharmashankar *et al.*, 2010; Feletou *et al.*, 2010b; Guimaraes *et al.*, 2001).

Studies on hypertensive or old animals demonstrate that their endothelial function is greatly altered (see elsewhere, Dharmashankar *et al.*, 2010; Feletou *et al.*, 2010b). Impairment of the EDH pathway (Brahler *et al.*, 2009; Weston *et al.*, 2010; Wu *et al.*, 1997) as well as the NO pathway (Kedziora-Kornatowska *et al.*, 2006; Kleinbongard *et al.*, 2006; Monti *et al.*, 2010; Schulz *et al.*, 2000) is known to exist alongside augmented EDCFs production during hypertension (Feletou *et al.*, 2009). On the other hand, raised levels of adrenaline are associated with hypertension in humans (Brown *et al.*, 1981; Goldstein, 1983), and in a hypertensive rat model high blood pressure was shown to be attributed to excessive activity of the sympathetic nervous system (Pinterova *et al.*, 2010). Even early studies have demonstrated that hypertension remains long after the injected adrenaline has disappeared from the blood plasma (Majewski *et al.*, 1981), whilst adrenergic agonists trigger release of EDRFs ex vivo (Figueroa *et al.*, 2009b; Figueroa *et al.*, 2001; Filippi *et al.*, 2001; Guimaraes *et al.*, 2001; Seya *et al.*, 2006). Despite extensive studies, the role that adrenoceptors may play in modulating endothelium-derived control of vascular tone remains to be understood.

1.6 Research aims

The experiments presented within this thesis were designed to evaluate the role of the endothelium in local and distant changes of vascular tone following activation of endothelium-dependent and -independent signalling pathways.

1. Signalling of β-adrenergic receptors causes relaxation and hyperpolarization of resistance arteries (Briones *et al.*, 2005; Garland *et al.*, 2011; Kozlowska *et al.*, 2003; White *et al.*, 2001), although the role of K_{ATP} channels in the functional responses that follow the receptor stimulation is not clear. Therefore, we aimed to clarify if signalling through the K_{ATP} channel contributes to local and conducted vasodilatation.
2. Vascular endothelium has been shown to express β-adrenoceptors (Briones *et al.*, 2005), although whether the endothelium participates in the dilatation to β-adrenergic agonist application by release of relaxing factors is a subject to debate. Therefore, we attempted to reveal a role that the endothelium may play in the β-adrenergic-mediated dilatation.
3. The levels of endogenous adrenergic agonists adrenaline and NA were shown to be increased during disease (Borkowski *et al.*, 1992; Brown *et al.*, 1981; Floras, 1992; Gayen *et al.*, 2010; Goldstein, 1983; Grassi *et al.*, 2010; Higashi *et al.*, 2002; Majewski *et al.*, 1981; Tung *et al.*, 1981), although whether adrenaline and NA can affect endothelial cell function is not known. Therefore, we decided to elucidate the effects of adrenergic agonists on the local and conducted dilatation to endothelium activation using the endothelium-dependent agonist ACh.

4. Together with EDH, NO is one of the most important endothelium-derived factors (Fleming *et al.*, 1999a). Recently, an important role for NO^- in rat vasculature was suggested (Irvine *et al.*, 2003a). We were interested in the signalling pathways of NO^--mediated dilatation and tested the hypothesis that endogenously produced NO^- may evoke a propagated response.

5. Endothelial function is known to be impaired in age and hypertension, leading to an imbalanced production of endothelium-derived relaxing and contracting factors (Feletou *et al.*, 2010b), whilst signalling of β-adrenoceptors is reduced (Borkowski *et al.*, 1992). The participation of EDCFs in responses to ACh and isoprenaline therefore were studied in small mesenteric arteries of young, old and old hypertensive rats.

Chapter 2. Methods

2.1 Rat mesenteric artery isolation and preparation

All experiments described in this thesis were performed using rat small mesenteric arteries. To obtain the arteries, young (12-14 weeks, 225-255g) or old (6 months, 550-650g) male Wistar rats were killed by cervical dislocation and exsanguination according to Schedule 1 of the Animals (Scientific Procedures) Act 1986 and monitored by the Home Office, UK. The mesenteric arcade was removed and placed in ice-cold MOPS buffered physiological solution (MOPS buffer, for salt composition see section 2.6) with pH adjusted to 7.40 ± 0.02 (at 37^{o}C). Part of the intestine together with its feeding vasculature was placed into a Sylgard-coated Petri dish filled with fresh ice-cold MOPS buffer (Figure 1B). Each mesenteric arcade was gently fixed with several small pins. Third-order branches of mesenteric artery were dissected free of adherent tissue under a dissecting microscope. Segments of the arteries, which contained no visible side branches, were cut to give a final length of ≥ 2 mm.

An external diameter of the arteries used in this work at 70 mmHg was between 220-340 μm for young rats and 351±18 (n=4) μm for mature animals.

2.2 Wire myography

Wire myography is a useful and convenient technique to perform in vitro studies of functional responses and vascular reactivity of vessels as small as 100 μm in diameter (Mulvany et al., 1977). First developed in 1972 by Bevan and Osher (Bevan et al., 1972), it was improved by Professors Mulvany and Halpern in 1976 (Mulvany et al., 1977). They secured both ends of each mounting wire with screws to fixed supports ('jaw') to keep the artery under tension, allowing the circumference of the vessels to be kept constant; therefore, the vessels were examined under near isometric conditions. Compounds were added directly to the chamber and vessel tension was monitored for contractile or relaxant responses.

Segments (2 mm long) of third-order mesenteric arteries of rat were mounted in a Mulvany-Halpern multi-channel myograph (Model 610M, DMT Denmark, Figure 1) using gold-plated tungsten wires (25 μm diameter) in Krebs solution (for salt composition see section 2.6). The arteries then were warmed up to 37 ºC and left to equilibrate for 20 minutes. Following the equilibration period, the arteries were normalised in order to obtain an optimal and standardized stretch according to Laplace's Law. For this, the internal diameter of the vessel was set to a resting tension equivalent to that generated at 0.9 times the diameter of the vessel at 70 mmHg.

After an additional 30 minutes of equilibration at the set resting tension, viability of the arteries were assessed by precontraction with phenylephrine (PE, 1-5 μM) and the following endothelium-dependent relaxation to ACh (0.1- 1 μM). Only arteries, which relaxed > 95% to 1μM ACh, displaying the functional endothelium, were used in subsequent experiments. To avoid effects of evaporation, bath solutions were changed each 20 minutes.

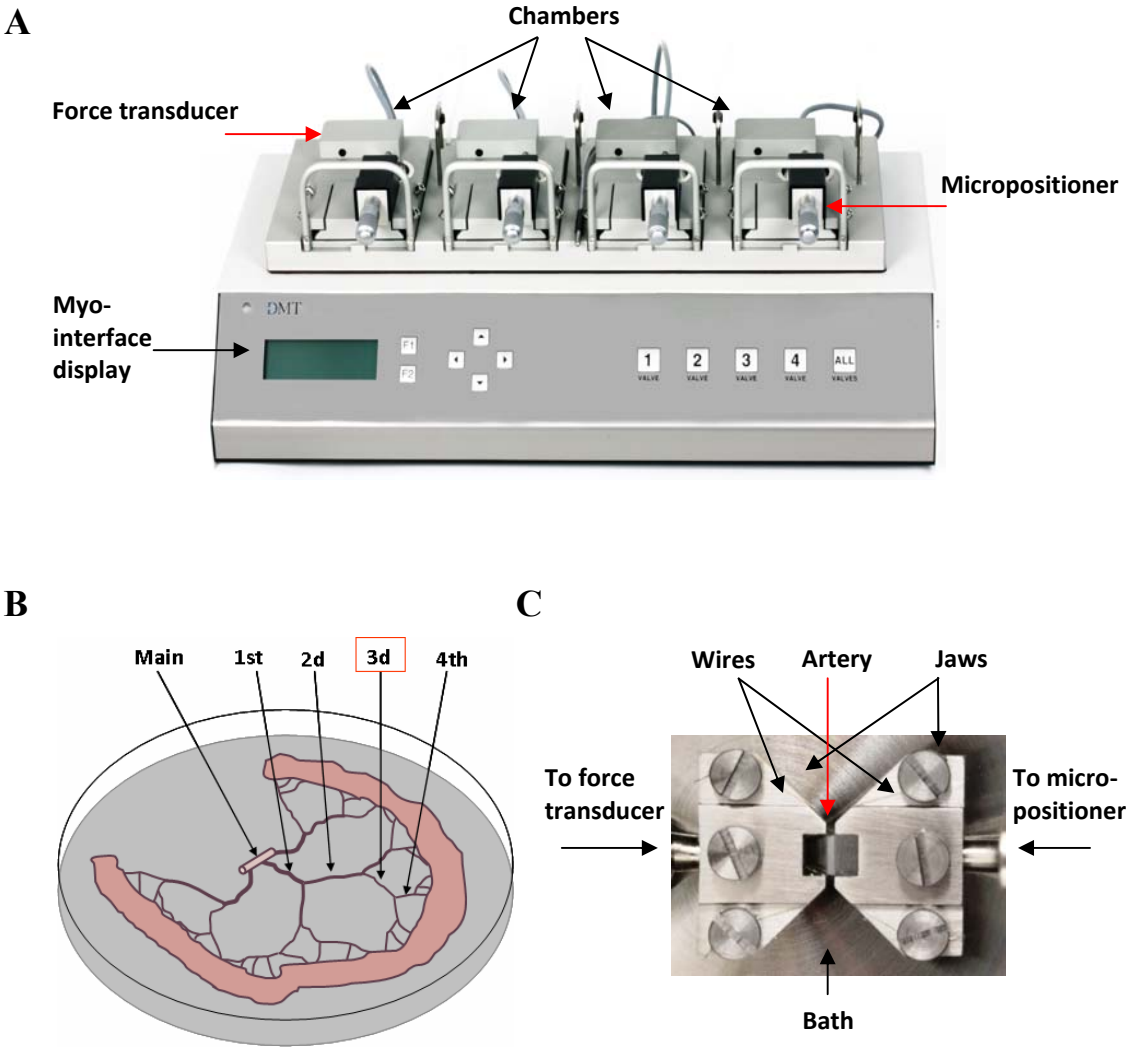


Figure 2.1 Experimental setup for wire myography and vessel mounting

A. Photograph of a front panel of the 4-channel wire myograph (DMT, Denmark), similar to the one used in this work*;
B. Schematic diagram of vascular bed with veins dissected away revealing arteries beneath. The vessel labelled Main is a main mesenteric artery, 1st, 2d, 3d, and 4th – are, subsequently, first, second, third and fourth order of mesenteric arteries. In wire myography experiments 3d order arteries were used.
C. Photograph of a third order rat mesenteric artery mounted in wire myograph jaw*.

* Photos are taken from DMT website: http://www.dmt.dk

2.2 Pressure myography

The internal elastic lamina together with the endothelial cell layer creates a barrier, which can hinder or even prevent the passive diffusion of molecules from blood plasma in and from surrounding tissue. To allow the possibility to change solution inside the vessel (luminal) independently of the external solution in the bath (abluminal) a pressure myography technique was used. Moreover, pressure myography allows studying responses of the arteries under isobaric conditions that may be more physiological than the wire myograph.

This technique was first developed in 1967 to study arterioles, up to 50 μm in diameter, perfused and pressurized through a single cannula (Uchida *et al.*, 1967), later the method was optimised to study microvessels as small as 12 μm (Duling *et al.*, 1981). Further improvements included continuous vessel imaging and introduced second cannula to allow changing luminal solution independently of abluminal (VanBavel *et al.*, 1990), importance of longitudinal stretching was emphasized (Coats *et al.*, 1999).

For pressure myography, all branches of the artery (two for local and three for spreading dilatation studies) were cannulated with glass pipettes, connected with gravity-fed pressure system. The preparation then was warmed up to 37°C and superfused with MOPS buffer for at least 20 min, then pressure, driven by gravity-fed inflow and outflow system, was gradually risen to 80 mm Hg. After the vessel was straightened and stretched, the pressure was subsequently decreased to 70 mm Hg and was maintained at this level during the whole experiment. Following 20 minutes of equilibration time, reactivity of the artery was assessed by precontraction of the artery with phenylephrine (PE) (0.5-3 μM) followed by endothelium-dependent relaxation to ACh (0.1 and 1 μM). Only vessels relaxing to 1 μM ACh by more than 95%, reflecting

viable endothelium, were subsequently used for further experiments. This method was used to study both local and distant (conducted) responses.

2.2.1 Luminal and abluminal application of agonists

During experiment, substances were either added directly into the bath (abluminally), or infused via the lumen (lumenally) by means of syringe pumps (BeeHive syringe pump system, Bioanalytical systems, USA). For luminal perfusion, one of the pipettes was disconnected from the gravity-fed pressurizing syringe reservoirs and attached to the syringe pump system. To obtain concentration-dependent responses, studied substances were added cumulatively, or non-cumulatively, when indicated. For the luminal application only five concentrations of agonist were used during one sequence of cumulative concentration response on each vessel. The speed of luminal flow did not exceed 50 μL/min for the initial phase of perfusion and 10 μL/min after the dead volume of the pipette was overcame. To check endothelial cell viability 1 μM ACh was applied at the end of each trace if relaxation was not absolute. 10 μM of levcromakalim was added at the end of experiments, when endothelial dependent dilatation of the artery was impaired.

2.2.2 Measurement of local responses

To study local responses, double cannulated vessels were used (Figure 2). For this, segments of arteries free from side branches were cut to give a final length of 3-4 mm, then cannulated with two glass pipettes (external diameter 150-200 μm) and positioned near the basement of a temperature-regulated chamber (10 mL, 120CP, Danish Myo Technology, Denmark) seated in a stage insert of an inverted microscope (IX71, Olympus). After equilibration, arteries where pressurized and stretched, and viability of the vessels was tested with PE and ACh, as described above (section 2.2).

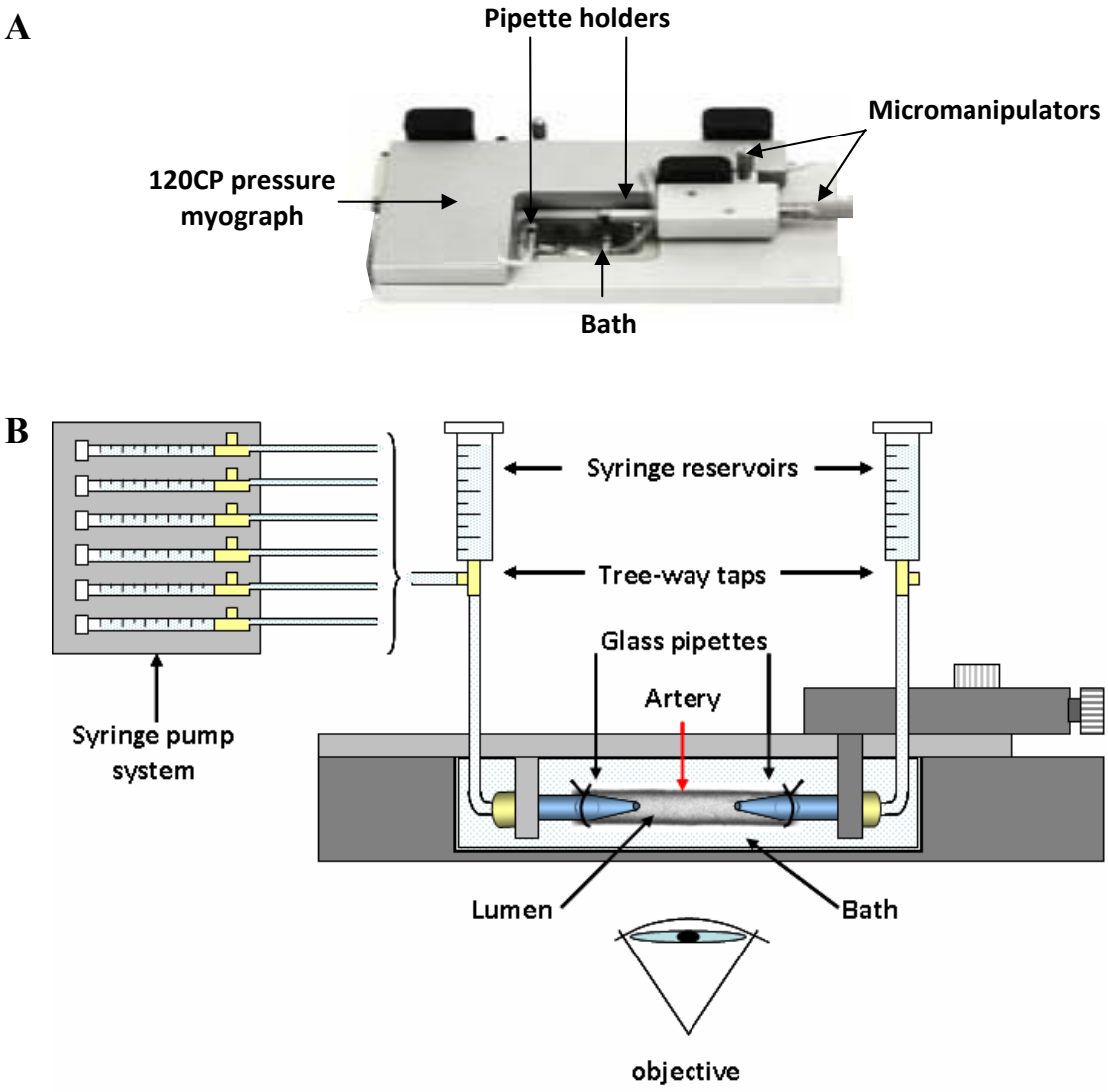


Figure 2.2 Experimental setup for studies of local responses

Isobaric diameter recordings by means of pressure myograph 120CP (DMT, Denmark) and system for luminal perfusion of agonists (BeeHive syringe pump system, Bioanalytical systems, USA).
A. A photograph illustrating the 120CP pressure myograph for double cannulated arteries*.
B. The diagram illustrates a cross-section of the setup. Artery is mounted on two pipettes in a 10 mL organ bath. One of the pipettes may be connected to syringe pump system, allowing luminal perfusion of studied compounds, whilst the other pipette is connected to a 5 ml syringe reservoir, raised to a calibrated height to exert intramural pressure of 70 mmHg.

* Photo is taken from DMT website: http://www.dmt.dk

The arteries were visualized using with 4 x/0.13 NA objective and cool snap CCD camera (KP-M1E/K-S10, Hitachi Kokusai Electric Inc., Japan) attached to the microscope. Vessel diameter changes were tracked by Vedi View software (v.1.2, Photonics Engineering).

2.2.3 Measurement of spreading responses

In experiments where conducted (or spreading) responses were studied, a recently developed method of triple cannulated vessels was used (Figure 3) (Winter *et al.*, 2007; Yuill *et al.*, 2009). For this, third order branch with a bifurcation (two forth order branches, subsequently) was isolated and all three ends were cannulated with glass pipettes (external diameter 100-200 μm), then the artery was positioned near the base of a 2 mL temperature-regulated chamber in a stage insert (RC-27 chamber, PH-6 platform, Warner Instruments, CT, USA), as previously described (Winter *et al.*, 2007). The arteries were subsequently pressurized, stretched and tested, as indicated above. The studied agonist was perfused into Branch 1 together with 10^{-7} M carboxyfluorescein; a constant flow of 1 μl/min was applied into Feed branch in order to restrict the studied agonist to Branches 1 and 2. Branch 2 served as an outflow for solutions derived from Branch 1 and Feed branch. Speed of the luminal flow did not exceed 5 μl/min during experiment. Additionally introduced superfusion flow (2 ml/minute) was constantly applied in the direction of flow inside the Feed branch.

In these experiments, brightfield images were captured together with fluorescence images (excitation 488 nm, emission 505 nm) to enable monitoring of agonist delivery. Using scanning confocal microscope (FV300-SU, Olympus, Japan) images were recorded with Fluoview Software (v.5.0, Olympus, Japan) at 1 Hz and processed with Metamorph software (Version 6.1, Universal Imaging, USA; Figure 4).

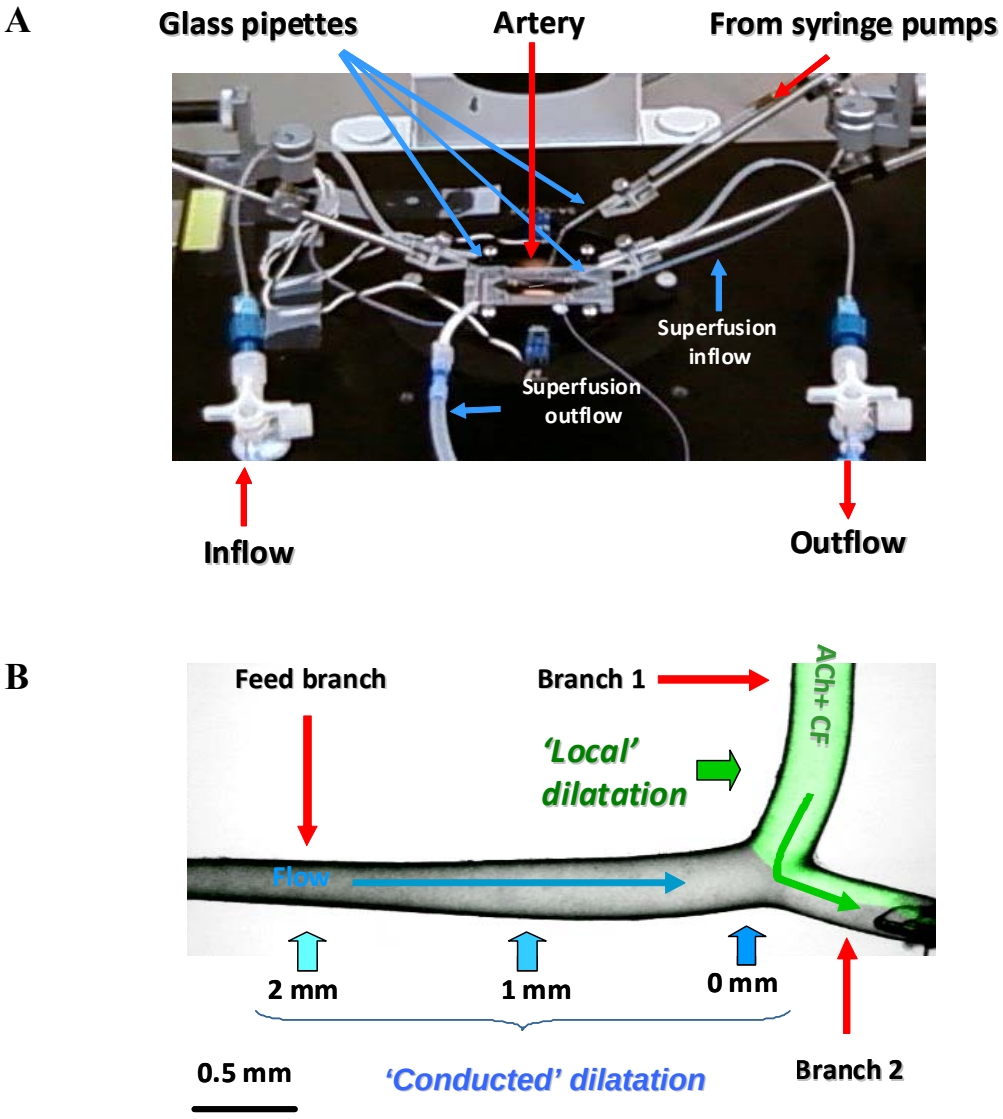


Figure 2.3 Experimental setup for studies of spreading responses

A. The figure shows a photograph illustrating the experimental setup for triple cannulated arteries. Arteries are mounted on three pipettes; two of the pipettes are connected to syringe pump system, allowing luminal perfusion of studied compounds, whilst the outflow pipette is connected to a 5 ml reservoir, raised to exert intramural pressure of 70 mmHg.
B. The diagram illustrates a triple cannulated artery. The studied agonist (here ACh) is perfused into Branch 1 together with carboxyfluorescein; a small constant flow is applied into Feed branch in order to restrict the studied agonist to Branches 1 and 2; Branch 2 serves as an outflow for solutions derived from Branch 1 and Feed branch. Additionally introduced superfusion flow is constantly applied in the direction of flow inside the Feed branch.

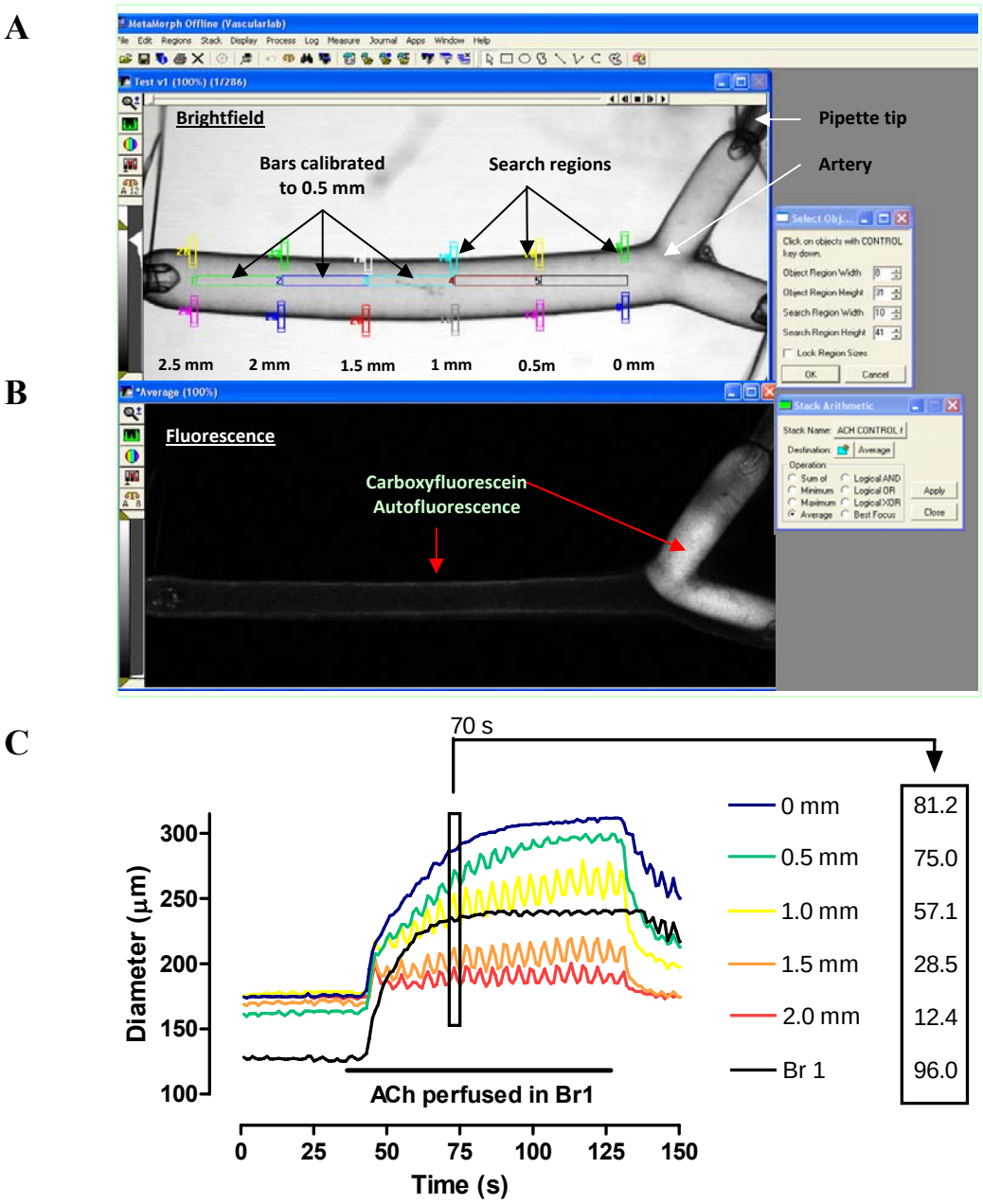


Figure 2.4 Analysis of spreading responses

A. The figure shows regions used for tracing artery motion (outer diameter) by the software program Metamorph (Universal Imaging, USA). Diameter measurements were performed at multiple sites along the Feed branch (distance between search regions is 0.5 mm) and at Branch1 (analyzed separately), using recorded time series from the brightfield channel. The 0 mm point represents nearest position to the edge of the stimulated area.

B. The figure represents an averaged image of the time series recorded at 505 nm, an emission wavelength of carboxyfluorescein added to the perfused solution together with agonist (stimulated area).

C. A representative trace illustrating the spread of dilatation in response to perfusion of ACh into Branch 1. The values for % maximum diameter at 70 s are shown in the box to the right, and correspond to each position along the artery.

2.4 Measurement of endothelial cell $[Ca^{2+}]_i$ in pressurized arteries

In separate experiments, small mesenteric arteries were dissected, double-cannulated and tested as described in section 2.1. Then the endothelial cells were loaded with Ca^{2+} dye, as shown previously (Kansui *et al.*, 2008; Rodriguez-Rodriguez *et al.*, 2009). Briefly, the pressure was lowered to 4 mm Hg and the artery was perfused with a filtered (0.22 μm pores) buffer containing 0.02% Pluronic F-127 and a cell-permeable Ca^{2+} dye Oregon Green 488 BAPTA-1 AM (10 μM) for 30 minutes to selectively load endothelial cells (Kansui *et al.*, 2008). After the dye was washed out with MOPS buffer, the pressure was re-introduced and artery was left for another 30 minutes for de-esterification. Using Olympus IV70 with Andor IQ 1.8.1 spinning disc microscope the sample was exited at 488 nm and emitted light was collected at 515 nm with 40x water immersion objective (UApo N340, Olympus, Japan). Endothelial cells were visualised in a clip box of 511x272 pixels to allow 10 Hz scan frequency. Cells in a good focus (6-10 cells) were selected and fluorescence intensity was determined off-line using Andor IQ 1.5 software. The data were then analysed and presented as (F/F_0), where F_0 is averaged basal intensity.

2.5 Data analyis

Data were analyzed using Microsoft Excel 2003 (Microsoft Corporation) and Graph Pad Prism (v4.03, GraphPad Software, USA) software. Relaxation is expressed as percentage of the maximal diameter of the artery ($\Delta D/\Delta D_{max}$) and contraction is expressed as percentage of the maximal contraction of the artery ($\Delta D/\Delta D_{min}$). Results are summarized as mean ± S.E.M. of *n* replicates, where *n* is number of arteries, each obtained from an individual animal. Concentration-response curves (CRCs) were fitted where possible using nonlinear regression, sigmoidal dose response function (variable slope):

$$Y = \frac{Bottom + (Top - Bottom)}{1 + 10^{(\log EC_{50} - X)Hill.Slope}}, \qquad \text{(Equation 3)}$$

where X is the logarithm of concentration; Y is the response; X starts at Bottom and goes to Top with a sigmoidal shape; EC_{50} is the concentration (M) of agonist that produces 50% of its maximum response. Agonist efficacy was expressed as the negative logarithm to base 10 of the EC_{50} value.

Spreading dilatation data were fitted using nonlinear regression, one-phase exponential decay:

$$Y = Span \cdot \exp(-K \cdot X) + Plateau \ , \qquad \text{(Equation 4)}$$

where $K=1/X_{mid}$, $Span=Y_{max}-Y_{min}$, $Plateau=Y_{min}$; starts at *Span*+*Plate*au and decays to *Plateau* with decay constant K.

Statistical analysis were performed using Student's *t*-test, one-way or two-way ANOVA analysis followed by Bonferroni post test. A value of $p < 0.05$ was considered to be statistically significant ($^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs. control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. treated).

2.6 Drugs and solutions

All drugs were obtained from Sigma (Poole, UK) with exception of apamin and iberiotoxin (Latoxan, France), forskolin (Biomol International), bisindolylmaleimide (BIS I; Calbiochem), Pluronic and Oregon green BAPTA-1 AM (Molecular Probes, UK) and Angeli's salt (odium trioxodinitrate), which was ordered from Axxora. U46619, TRAM-34 and forskolin were dissolved in dimethyl sulfoxide, indomethacin was dissolved in 5% $NaHCO_3$, adrenaline and noradrenaline bitartrate salts were dissolved in 10^{-4} M ascorbic acid, iberiotoxin was reconstituted in physiological buffer and Angeli's salt was dissolved in 10^{-2} M NaOH to prevent decomposition prior to use (Favaloro *et al.*, 2009). L-cysteine was added to a specially prepared MOPS buffered solution with reduced amount of NaCl in it to balance excess of Na^+ incoming with NaOH (to reach pH=7.4). To get saturated solution of $NO^{\bullet}$ with NO concentration of 1.9 mM (Zacharia *et al.*, 2005), de-gassed MOPS buffered solution was bubbled with NO gas (Favaloro *et al.*, 2009; Friedemann *et al.*, 1996; Rajanayagam *et al.*, 1993; Simonsen *et al.*, 1999). All other stock solutions were prepared using MilliQ water. All stock solutions were 10^{-2} M, except of L-NAME 10^{-1} M, and were diluted to working concentrations in physiological buffer before the experiment. Inhibitors were pre-incubated with the tissue for at least 20 minutes before agonist application.

Krebs buffer contains (in mM): NaCl, 118.0, $NaCO_3$, 25; KCl, 3.6; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; glucose, 11.0; $CaCl_2$, 2.5; and gassed with 95 % O_2 and 5 % CO_2 before and during experiment.

MOPS buffer contains (in mM): NaCl, 145; KCl, 4.7; $CaCl_2$, 2.0; $MgSO_4 \cdot 7H_2O$, 1.17; MOPS, 2.0; $NaH_2PO_4 \cdot H_2O$, 1.2; glucose, 5.0; pyruvate, 2.0; EDTA, 0.02; NaOH, 2.75; with pH adjusted to 7.40 ± 0.02 (at 37^{o}C).

Chapter 3. The role of K_{ATP} channels in β-adrenoceptor-mediated vasodilatation

3.1 Introduction

More than a century ago it was reported that vasoconstriction occurs following injection of adrenal gland extracts (Oliver *et al.*, 1895). Later it was found that adrenaline, the compound responsible for the observed phenomenon, can also evoke vasodilatation in the presence of the alkaloid ergotoxine (Dale, 1913). Observations such as these led to the discovery of two main groups of adrenoceptors: α-adrenoceptors and β-adrenoceptors (Ahlquist, 1948), with the subsequent development of a selective α-adrenoceptor antagonist phentolamine (Urech *et al.*, 1950) and β-adrenoceptor antagonist propranolol (Black *et al.*, 1965). Each adrenoceptor group was further subdivided into several subtypes, such that nine subtypes of the receptor are now recognized. α_1-adrenoceptors are coupled to G_q protein, which stimulates phospholipase C and through elevation of $[Ca^{2+}]_i$ causes vasoconstriction, while α_2-adrenoceptors are linked to G_i protein and decrease $[cAMP]_i$. All β-adrenoceptor subtypes stimulate adenylyl cyclase and enhance cAMP production, and in the vasculature this leads to vasodilatation (for review see Guimaraes *et al.*, 2001).

In the mesenteric vascular bed, the contractile response of smooth muscle to catecholamines is mediated predominantly through the α_1-adrenoceptor (Hussain *et al.*, 2000; Piascik *et al.*, 1997). The subtype of β-adrenoceptors that is responsible for dilatation in rat mesenteric arteries remains unclear. Whilst a leading role for the β_2-adrenoceptor was suggested initially (Kozlowska *et al.*, 2003), others demonstrated less sensitivity to the β_2-adrenoceptor agonist salbutamol and suggested a predominant contribution of β_1-adrenoceptors to vasodilatation (Briones *et al.*, 2005). On the other hand, participation of both receptor subtypes in the response to β-adrenoceptor agonists was previously shown for this vascular bed (Zwaveling *et al.*, 1996). Further studies of

the propranolol-resistant component of the isoprenaline-induced arterial dilation revealed the involvement of a third, atypical subtype of the β-adrenoceptor family (Kozlowska *et al.*, 2003; Sooch *et al.*, 1995), possibly the β_3-subtype (Dessy *et al.*, 2004; Figueroa *et al.*, 2009b).

Stimulation of β-adrenoceptors was also associated with smooth muscle cell hyperpolarization (Holman *et al.*, 1968; Prehn *et al.*, 1983; Somlyo *et al.*, 1970), which was a result of the adenylyl cyclase-dependent activation of K^+ channels, predominantly the K_{ATP} channel (Fujii *et al.*, 1999; Nakashima *et al.*, 1995; Somlyo *et al.*, 1970). Although cAMP signalling also involves stimulation of Epac (Gloerich *et al.*, 2010), it has been shown that Epac activation inhibits K_{ATP} channel activity (Purves *et al.*, 2009), whilst the stimulatory pathway involves phosphorylation of the SUR2 subunit of the K_{ATP} channel by PKA (Shi *et al.*, 2007).

Recent experiments by our group have demonstrated that both classical β-adrenoceptor subtypes could evoke hyperpolarization and dilatation in rat small mesenteric arteries (Garland *et al.*, 2011). The principal route for hyperpolarization was via β_1-adrenoceptors, with β_2-adrenoceptors playing less of a role, whereas β_3-adrenoceptor stimulation failed to induce any change in membrane potential. In both cases, the K_{ATP} channel antagonist glibenclamide was sufficient to suppress the hyperpolarization.

Although hyperpolarization of vascular tissue is generally associated with dilatation (Waldron *et al.*, 1994), it is not clear to what extent the K_{ATP} channels contribute to the functional response of β-adrenergic stimulation, particularly in response to physiological agonists such as noradrenaline (NA) and adrenaline.

One of the established essential characteristics of resistance arteries is the ability to synchronise responses within a certain length of the vessel, which reflects the

electrochemical coupling of the vascular cells through the myoendothelial gap junctions (Dora, 2010; Figueroa *et al.*, 2009b; Garland *et al.*, 2010a). It has been recently reported that the hyperpolarization to the K_{ATP} channel opener levcromakalim can propagate along the wall of the mesenteric artery and evoke distant vasodilatation (Takano *et al.*, 2004). Thus, we hypothesized that β-adrenoceptor-mediated activation of the K_{ATP} channels may initiate a similar spread of dilatation, termed 'conducted dilatation'.

Therefore, the goal of this part of my study was to examine the role of K_{ATP} channels in the local and conducted response to adrenoceptor stimulation, and evaluate the receptor subtypes involved and the pathways of propagation of the response in rat small mesenteric arteries.

3.2 Methods

3.2.1 Rat mesenteric artery isolation and preparation

See section 2.1 for detailed description of artery isolation and preparation.

3.2.2 Wire myography

The arterial segments were mounted in a Mulvany-Halpern 4-channel myograph (Model 610M, DMT Denmark) and normalized as described in section 2.2. Artery viability was examined by pre-contraction with the α-adrenergic agonist phenylephrine (PE, 1-10 μM) and > 95% relaxation to ACh (0.1-1 μM).

3.2.3 Pressure myography for the spreading dilatation studies

The pressure myograph system was employed to study the spreading responses. To enable selective stimulation of one part of the vessel, rat small mesenteric arteries possessing a bifurcation were cannulated and pressurized as described in section 2.2. To obtain denuded arteries, the pressure was decreased and an air bubble (~2.5 mm^3) was perfused through the artery using a syringe pump system. The lumen was then washed for 5 minutes and 70 mm Hg pressure was re-introduced.

3.2.4 Measurement of the spreading responses

The protocol for the spreading dilatation studies is described in section 2.2.3. Figure 3.1 demonstrates a diagram of the triple cannulated vessels and the principle of the spreading dilatation measurements.

3.2.5 Cumulative concentration-response curves

Increasing concentrations of the studied agonists were introduced directly to the bath following submaximal (70-80% of maximal tone) precontraction with PE. A stable TP receptor agonist U46619 was used to achieve the tone when α-adrenergic inhibitors had been applied to reveal β-adrenoceptor-mediated responses. In the presence of the K_{ATP} channel inhibitor glibenclamide (5 μM) the concentration of U46619 was increased in order to reach similar level of tone as in the control (from 32.3 ± 1.9 nM to 728.4 ± 133.9 nM, n = 4). This may be explained by an antagonistic effect of glibenclamide on the TP receptor established for rat arteries (Kemp *et al.*, 1998).

3.2.6 Data analysis

The data were analyzed as described previously (see section 2.5).

3.2.7 Drugs and solutions

See section 2.6 for the preparation details.

Krebs buffer, continuously gassed with 95% O_2 and 5% CO_2, was used for the wire myograph experiments, whilst MOPS buffered solution was used for the conducted dilatation studies.

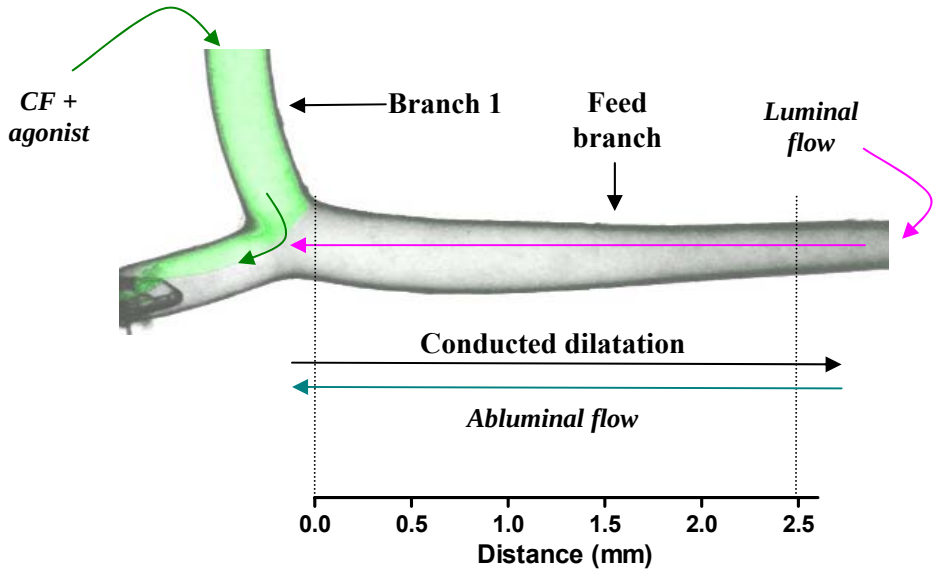


Figure 3.1 Diagram illustrating triple cannulated segment of artery for measurements of conducted dilatation responses

The agonist was infused via Branch 1 together with carboxyfluorescein (CF). Luminal flow (1-2 µl/min) inside the Feed branch (magenta arrow) restricted flow of the studied agonist within the bifurcation. Abluminal flow (2 ml/min) was introduced in the direction opposite of the direction of conducted response (blue arrow) to prevent agonist diffusion upstream from the point of bifurcation. Diameter was measured in Branch 1 and at 0.5 mm increments from the edge of applied agonist (evaluated by CF) along the Feed branch.

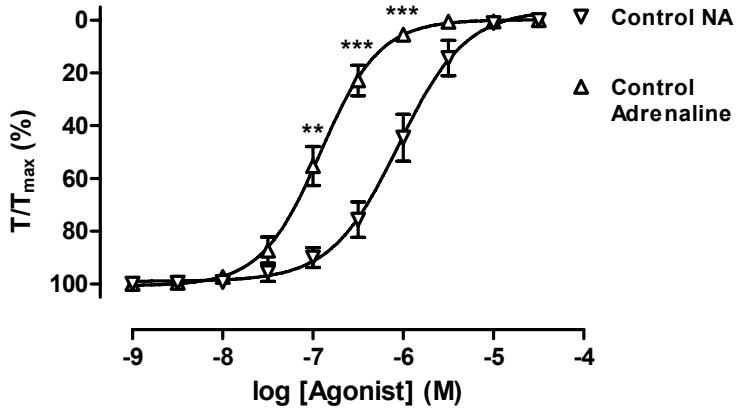
3.2 Results

3.2.1. Effects of adrenaline and noradrenaline on tension

There is evidence that stimulation of adrenoceptors may lead to release of nitric oxide (NO) in rat mesenteric arteries (Figueroa *et al.*, 2009b), therefore all experiments in this section were performed in the presence of NOS inhibitor L-NAME (100 μM).

Adrenaline and NA constricted rat small mesenteric arteries in a concentration-dependent manner (Figure 3.2A). Adrenaline was a significantly more potent vasoconstrictor than NA (pEC_{50} = 6.92 ± 0.05, n = 14 and pEC_{50} = 6.05 ± 0.08, n = 14, respectively, p<0.05). The α_1-adrenoceptor antagonist prazosin (1 μM) blocked the contraction and revealed a concentration-dependent dilatation to adrenaline and NA in arteries preconstricted with the thromboxane mimetic U46619 (Figure 3.3B). Though the relaxation to adrenaline tended to have a higher efficacy than NA, the difference was not statistically significant (pEC_{50} = 6.33 ± 0.09, n = 10 and pEC_{50} = 5.93 ± 0.07, n = 10, respectively, p>0.05). Treatment with the classical β-adrenoceptor antagonist propranolol (1 μM) resulted in unaltered contraction to adrenaline (from pEC_{50} = 7.09 ± 0.06 to pEC_{50} = 7.04 ± 0.05, n = 8, p>0.05) and NA (from pEC_{50} = 6.32 ± 0.06 to pEC_{50} = 6.48 ± 0.07, n = 8, p>0.05), but dramatically suppressed the vasodilatation to adrenaline (from pEC_{50} = 6.64 ± 0.05 to pEC_{50} = 4.11 ± 2.3, n = 6, p<0.05) and NA (from pEC_{50} = 6.18 ± 0.06 to pEC_{50} = 5.09 ± 0.08, n = 6, p<0.05), indicating that β-adrenoceptors are the predominant receptors involved in the vasodilatation to the nonselective adrenergic agonists (Figure 3.3A,B).

A



B

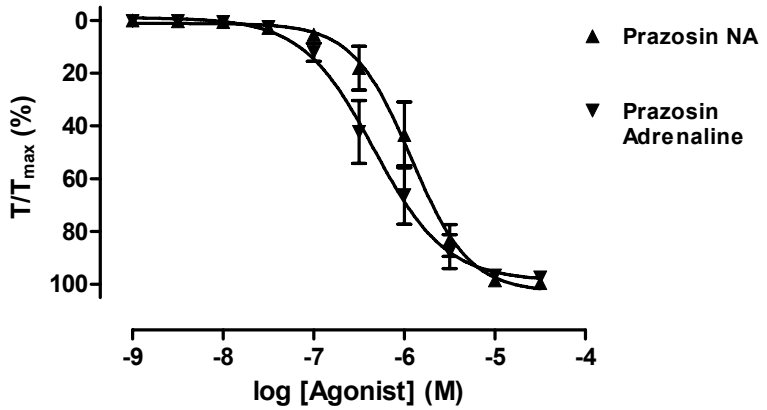
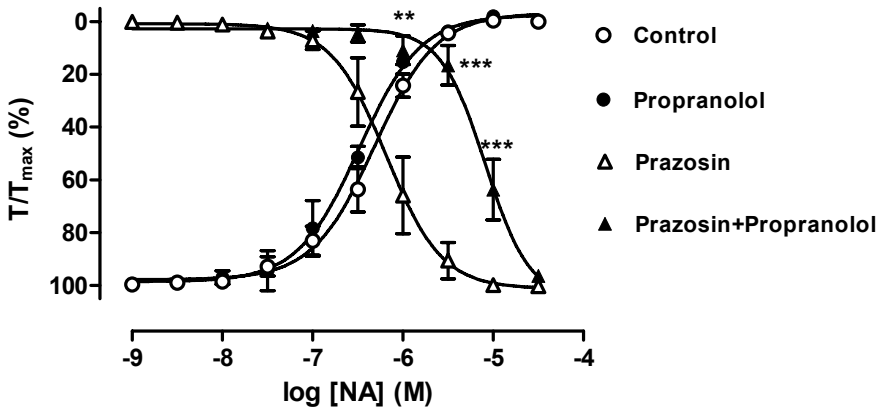


Figure 3.2 Concentration-response curves to adrenaline and NA in small mesenteric arteries mounted in wire myograph.

A. Summarized data showing the concentration-dependent contraction to adrenaline and NA (n = 14, p<0.05);
B. Summarized data showing concentration-dependent relaxation to adrenaline and NA in the presence of α_1-adrenoceptor inhibitor prazosin (1 μM; n = 10, p>0.05). Arteries were submaximally preconstricted using the TP receptor agonist U46619.

Results shown are the mean ± s.e.mean, **p<0.01, ***p<0.001 vs. NA.

A



B

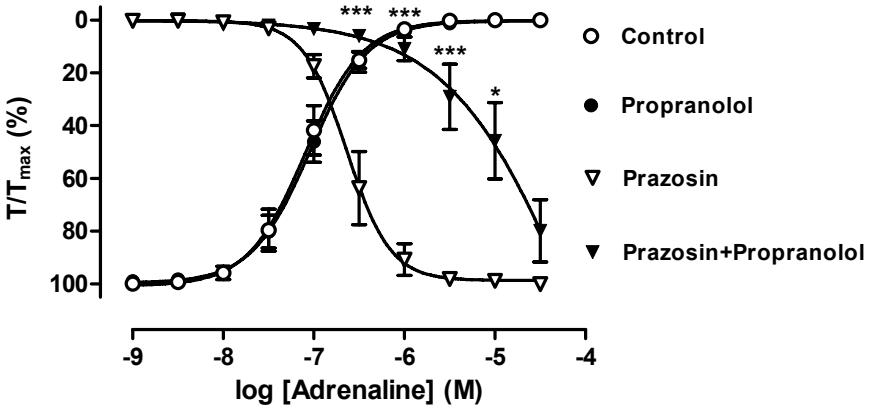


Figure 3.3 Effect of propranolol on the concentration-response curves to adrenaline and NA of small mesenteric arteries mounted in wire myograph

A. Summarized data showing contraction and relaxation of the artery to rising concentrations of NA. In control β-adrenergic antagonist propranolol (1 μM) had no effect (n = 8, p>0.05), but when the vessel was pre-treated with α_1-adrenoceptor inhibitor prazosin (1 μM) and precontracted with thromboxane mimetic U46619, propranolol significantly suppressed the response (n = 6, p<0.05).
B. Similarly, whilst propranolol was ineffective in control (n = 8, p>0.05), it affected the relaxation to adrenaline in the presence of prazosin following precontraction with U46619 (n = 6, p<0.05).

Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01 ***p<0.001 vs. prazosin.

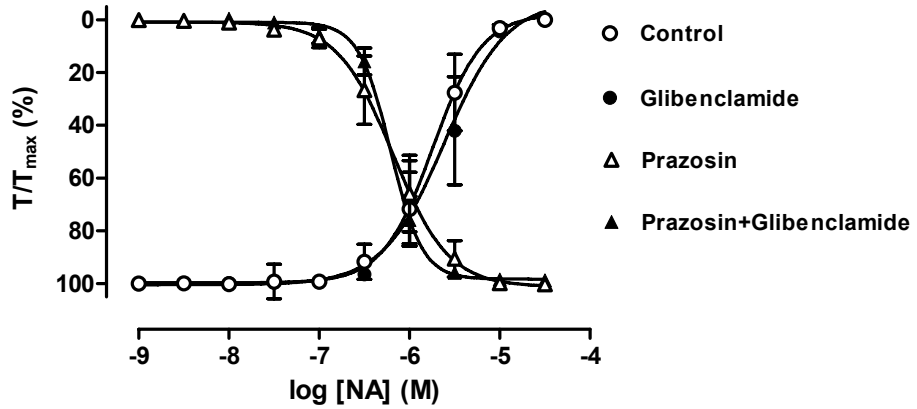
3.2.2. Participation of K_{ATP} channels in the relaxation mediated by β-adrenoceptor stimulation

It is known that stimulation of β-adrenoceptor leads to K_{ATP} channel-mediated hyperpolarization of smooth muscle cells (Hussain *et al.*, 2000; Piascik *et al.*, 1997); however, is not clear to what extend this may contribute to the functional response. Therefore we decided to establish the role of K_{ATP}-channel in the vasomotor responses by means of the K_{ATP}-channel inhibitor glibenclamide (5 μM). Glibenclamide failed to significantly modify both contraction and relaxation to adrenaline (contraction: from pEC_{50} = 6.71 ± 0.06 to pEC_{50} = 6.5 ± 0.07, n = 6-7, p>0.05; relaxation: from pEC_{50} = 6.64 ± 0.05 to pEC_{50} = 6.59 ± 0.07, n = 3-6, p>0.05) and NA (contraction: from pEC_{50} = 5.74 ± 0.03 to pEC_{50} = 5.61 ± 0.14, n = 6, p>0.05; relaxation: from pEC_{50} = 6.18 ± 0.08 to pEC_{50} = 6.21 ± 0.03, n = 5-6, p>0.05), indicating that the other mechanisms, distinct from the hyperpolarization via K_{ATP}-channel are responsible for the relaxation to the β-adrenoceptor stimulation (Figure 3.4A,B).

3.2.3. Influence of the agonist used to obtain tone on the endothelium-dependent relaxation to ACh

ACh activates endothelial muscarinic M_3 receptor in rat mesenteric arteries (Fujimoto *et al.*, 1991; Rodriguez-Rodriguez *et al.*, 2009; Wu *et al.*, 1997). Arteries precontracted with PE dilated progressively to rising concentrations of ACh (1 nM - 3 μM; pEC_{50} = 7.28 ± 0.02, n = 7). Precontraction with NA did not alter the response to ACh (pEC_{50} = 7.3 ± 0.04, n = 6; Figure 3.5A), whereas precontraction with adrenaline (pEC_{50} = 6.72 ± 0.04, n = 5, p<0.05; Figure 3.5A) or U46619 (pEC_{50} = 6.93 ± 0.02, n = 6, p<0.05; Figure 3.5B) resulted in the significant inhibition of ACh-induced dilatation.

A



B

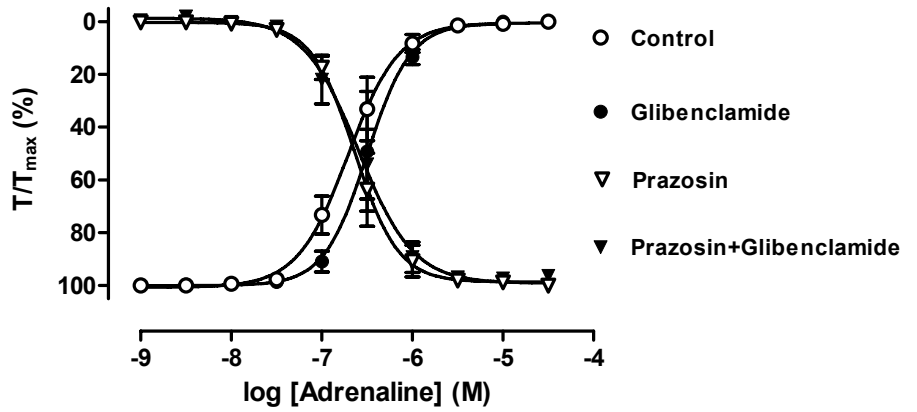


Figure 3.4 Effect of glibenclamide on the concentration-response curves to adrenaline and NA in small mesenteric arteries mounted in the wire myograph

A. Summarized data demonstrating contraction and relaxation to rising concentrations of NA. In control as well as in the presence of the thromboxane mimetic U46619 and the α-adrenoceptor inhibitor prazosin (1 μM), K_{ATP} channel inhibitor glibenclamide (5 μM) did not alter the response (n = 5 – 6, p>0.05).
B. Glibenclamide was also unable to alter the concentration-response curve to adrenaline obtained in control (n = 3 – 6, p>0.05), or following treatment with prazosin and precontraction with U46619 (n = 6 – 7, p>0.05).

Results shown are the mean ± s.e.mean.

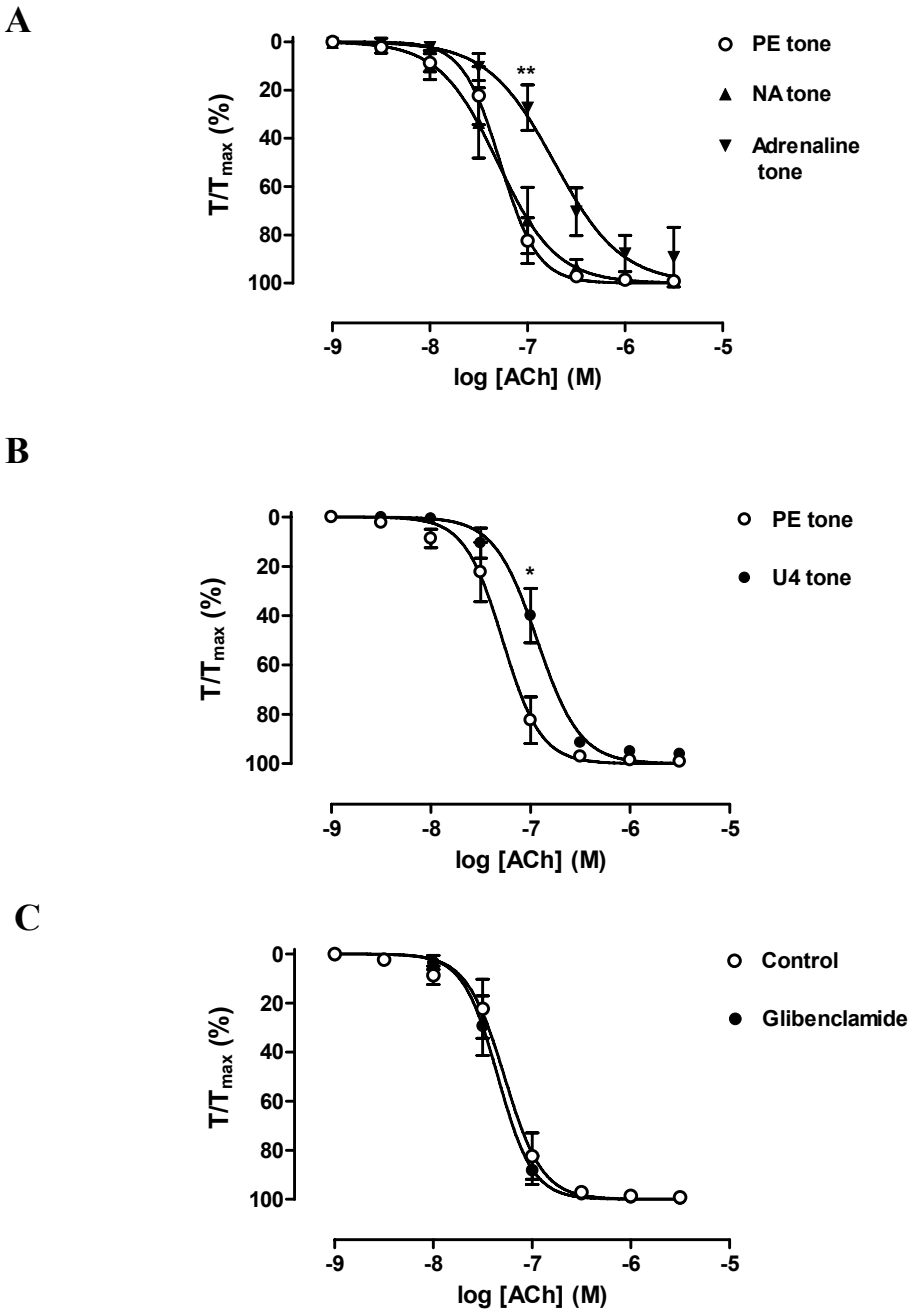


Figure 3.5 Effects of contractile agonists or glibenclamide on the relaxation to ACh in small mesenteric arteries mounted in wire myograph

A Precontraction of rat mesenteric arteries with the nonselective adrenoceptor agonist adrenaline resulted in a rightward shift of the concentration-response curve to ACh (1 nM – 3 μM, n = 5, p<0.05), when compared with the tone induced by α_1-adrenoceptor agonist PE (n = 7, p>0.05), whilst NA (n = 6, p>0.05) did not display such an inhibitory effect on the relaxation to ACh.
B Precontraction with TP receptoragonist U46619 (U4) also suppressed the relaxation evoked by ACh (n = 6 – 7, p<0.05).
C K_{ATP} channel inhibitor glibenclamide (5 μM) did not affect the concentration-response curve to ACh (n = 7, p>0.05).
Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01 vs. PE tone.

3.2.4. Conducted vasomotor response to adrenaline

In order to investigate whether adrenergic stimulation is able to evoke a conducted vaosdilatation, we infused the adrenoceptor agonist adrenaline (1 μM) into Branch 1 of the triple-cannulated pressurized small mesenteric arteries (Figure 3.1). Adrenaline constricted the artery at the site of application (to 31.3 ± 10.8% of the maximal tone); however, no conducted vasoconstriction was observed in the Feed branch (n = 4; Figure 3.6C). Interestingly, when the arteries were preconstricted to 58.5% ± 5.3 of the maximal tone with the abluminal application of U46619, perfusion of adrenaline into Branch 1 resulted in an enhanced local contraction (51.2 ± 5.0%) and appearance of spreading dilatation along the Feed branch (n = 5, p<0.05; Figure 3.6A,C). The vasodilatation reached 24.5 ± 2.9% at the "zero" point (the nearest point to the Branch 1) and had little decay at 2.5 mm upstream (17.1 ± 3.9%). The spreading dilatation was blocked by propranolol (1 μM), confirming involvement of β-adrenoceptors in the observed phenomenon (n = 5, p<0.05; Figure 3.6C).

3.2.5. Participation of the endothelium in the conducted vasodilatation to isoprenaline

To determine the role of the endothelium in the spreading response to β-adrenoceptor stimulation we have used β-adrenoceptor agonist isoprenaline (1 μM). In arteries preconstricted with U46619, isoprenaline induced a marked local dilatation followed by a spreading response along the Feed branch, which was similar to the response, induced by ACh (n = 9; Figure 3.7A). Whilst the denudation of the artery resulted in the loss of both local and conducted responses to ACh (n = 2, p<0.05; Figure 3.7B), removal of the endothelium inhibited only the conducted vasodilatation to isoprenaline, whereas the local response remained unaltered (n = 4; Figure 3.7C).

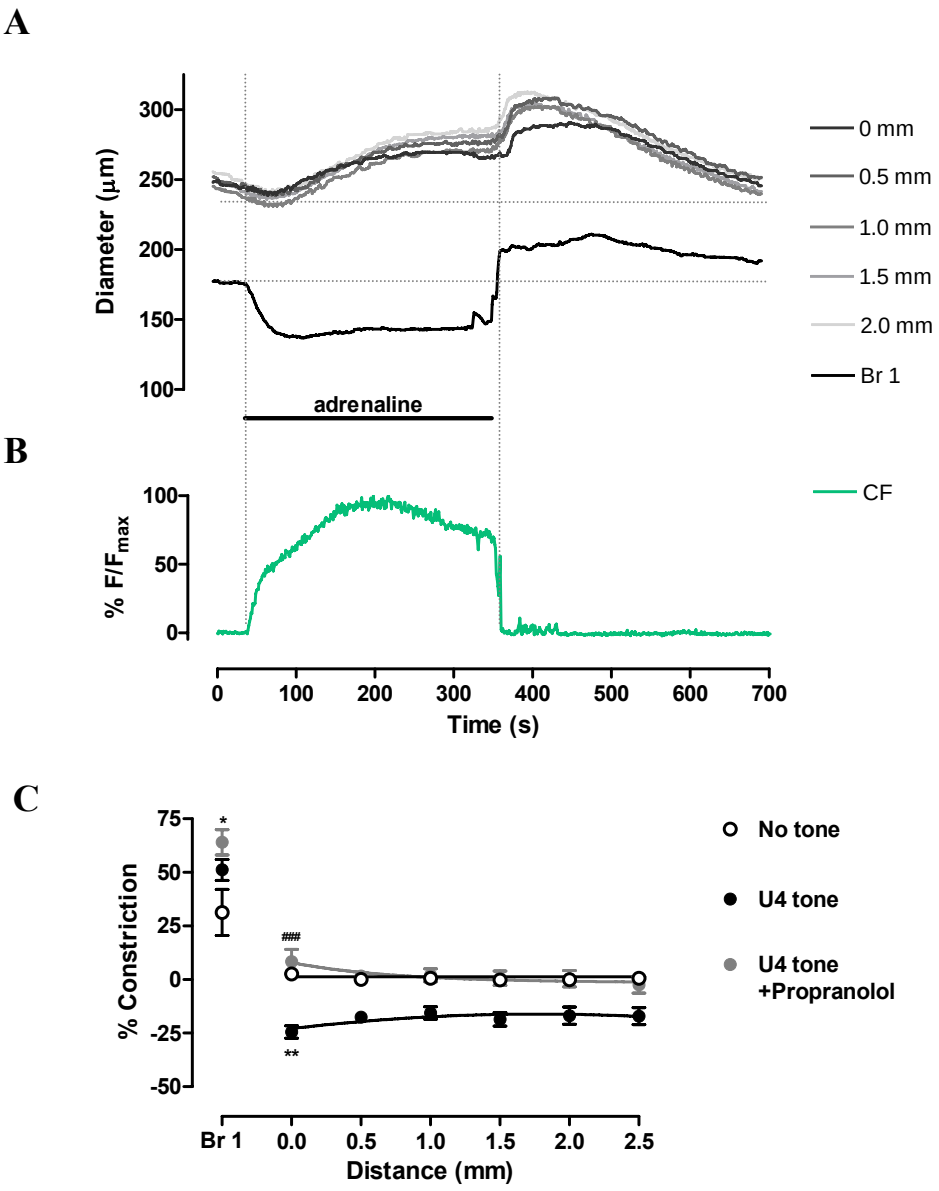


Figure 3.6 Conducted vasomotor response to adrenaline in triple-cannulated pressurized small mesenteric arteries

A. Representative trace illustrating conducted response to luminal perfusion of adrenaline (1 µM) into Branch 1. While Branch 1 displayed vasoconstriction, conducted dilatation was observed in Feed branch.
B. Change in intensity of carboxyfluorescein (CF) fluorescence measured in Branch 1 correlates with the delivery of adrenaline.
C. Summarized data showing conucted responses to local application of adrenaline at rest (no tone), after pre-constriction with thromboxane mimetic U46619 (~50% of max tone) in control conditions, and after pre-treatment with β-adrenoceptor antagonist propranolol (1 µM; n = 3-4, $p<0.05$).

Results shown are the mean ± s.e.mean;, *$p<0.05$, **$p<0.01$vs. 'no tone', $^{###}p<0.001$ vs. 'U4 tone'.

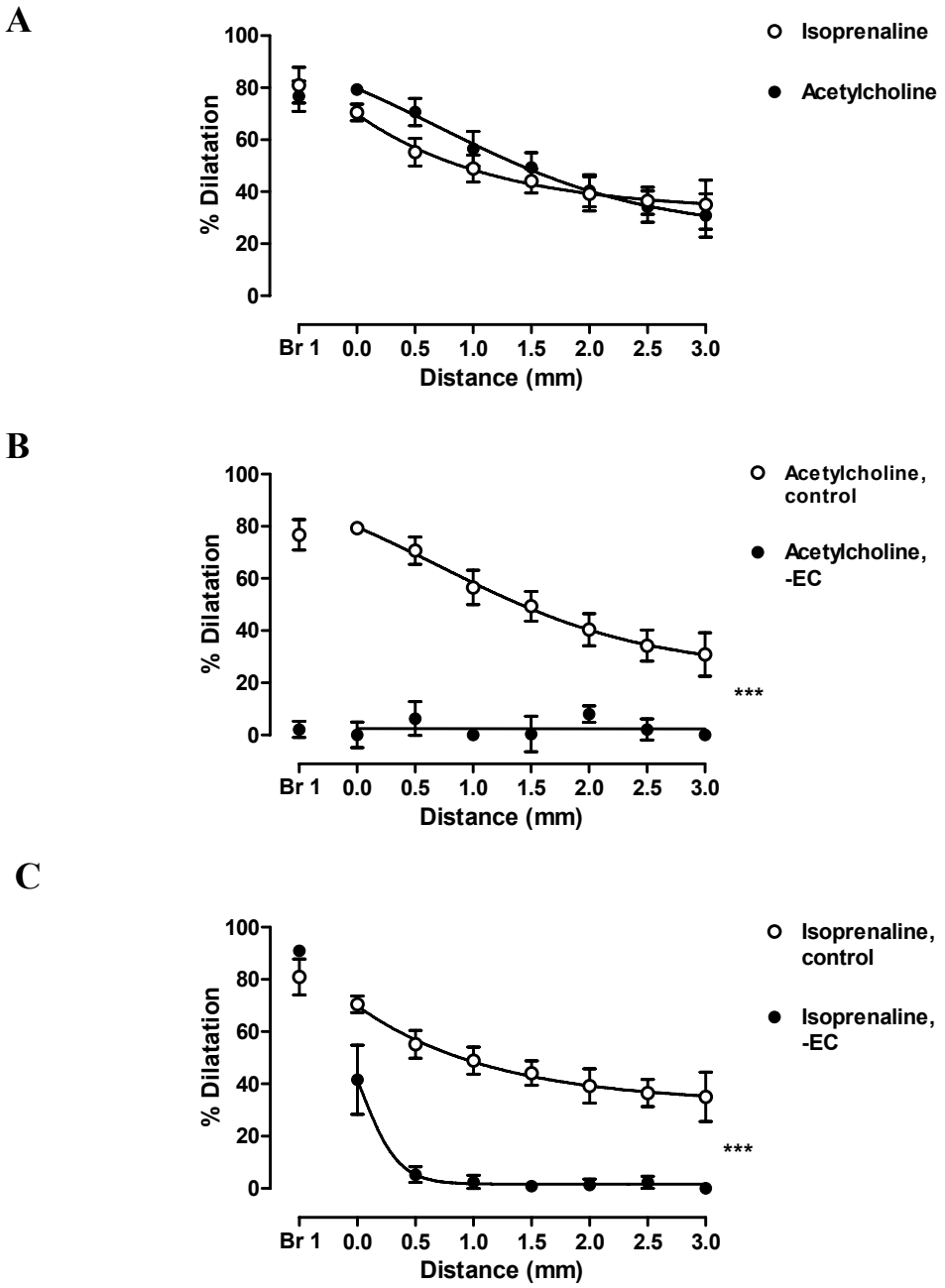


Figure 3.7 Endothelium-dependent and –independent conducted dilatation in triple-cannulated pressurized small mesenteric arteries

A. Summarized data illustrating conducted dilatation responses to luminal application of muscarinic cholinergic receptor agonist ACh (1 μM) and β-adrenergic agonist isoprenaline (1 μM) in arteries preconstricted with U46619 ($n = 9$).
B. Effect of endothelium removal on the conducted dilatation to ACh ($n = 2$).
C. Effect of endothelium removal on the spreading dilatation to isoprenaline ($n = 4$).
Results shown are the mean ± s.e.mean; ***$p<0.001$ vs. control.

3.2.6. Adrenoceptor subtypes responsible for the spreading dilatation

In this series of experiments prazosin was used to reveal vasodilatation of the arteries submaximally preconstricted with U46619, to adrenaline and NA. Perfusion of adrenaline (1 μM) and NA (1 μM) into Branch 1 resulted in the local dilatation (75.1 ± 6.3% and 56.1 ± 10.8%, subsequently, *n* = 7) followed by spreading dilatation upstream in the Feed branch (Figure 3.8). Inhibition of α_2-adrenoceptors with yohimbine (1 μM) resulted in an enhanced local response (90.6 ± 7.1%, *n* = 6 and 72.7 ± 7.1%, *n* = 7, respectively), whilst its co-application with propranolol inhibited the local dilatation (42.5 ± 9.3%, *n* = 5, and 35.8 ± 6.3 %, *n* = 6, respectively), which was further suppressed by the addition of β_3-adrenoceptor antagonist SR 59230A (1 μM, 16.0 ± 15%, *n* = 3, and 14.5 ± 7.8%, *n* = 6, respectively; Figure 3.8B,C). The amplitude of the conducted response matched the magnitude local dilatation remained after application of adrenergic receptor antagonists (Figure 3.8A,B).

3.2.7. Participation of K_{ATP} channels in the conducted dilatation to β-adrenoceptor stimulation

In this section we examined the hypothesis that although K_{ATP} channels do not play a role in the local dilation they may contribute to the conducted vasodilatation to β-adrenoceptor stimulation. In the presence of glibenclamide (10 μM) the local dilatation to adrenaline (*n* = 5), NA (*n* = 5), or isoprenaline (*n* = 5, not shown) did not spread from the site of application more than 0.5-1 mm upstream in the Feed branch (*n* = 5; Figure 3.9A,B), whilst the conducted vasomotor response to ACh was unaffected (*n* = 5; Figure 3.9C).

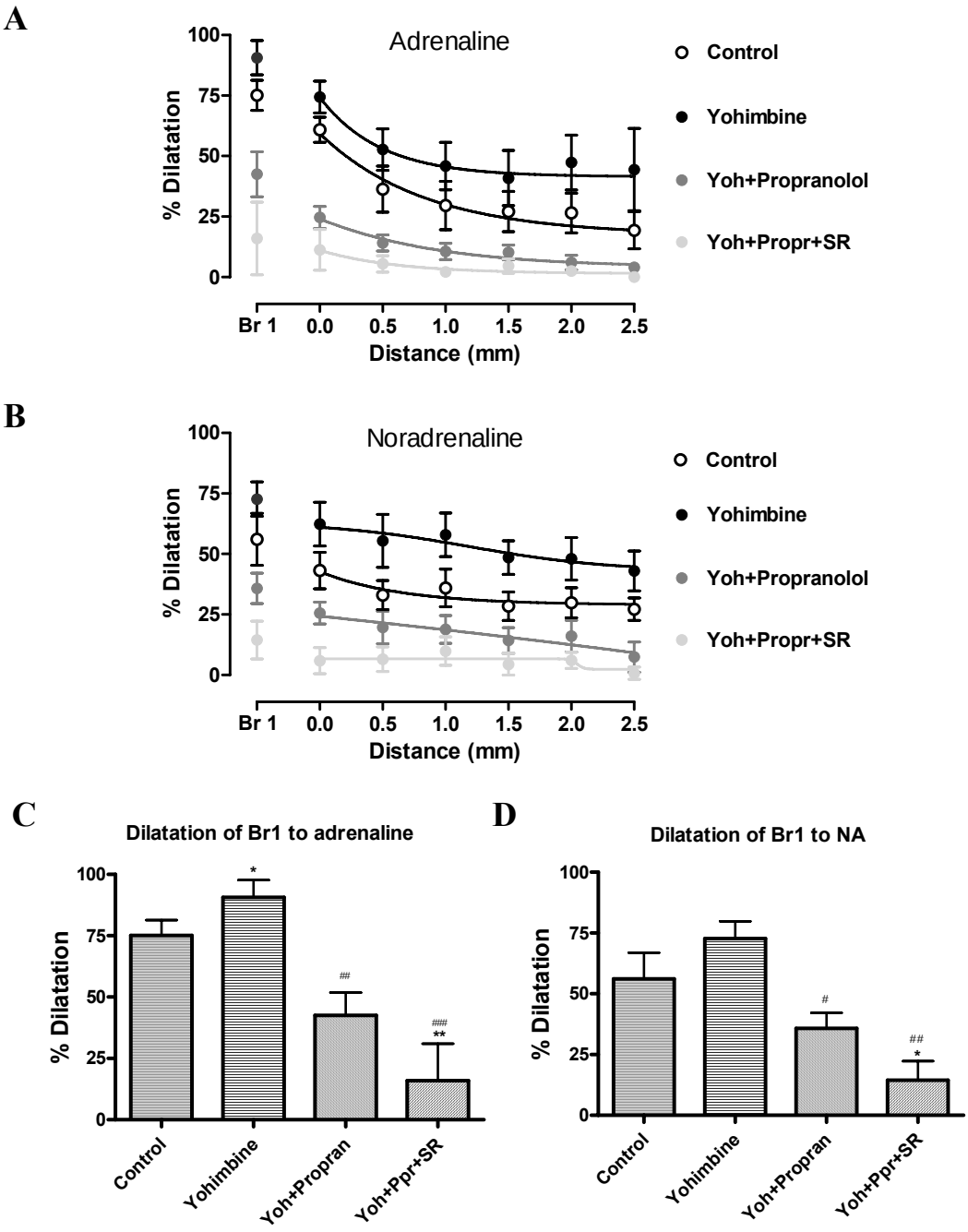


Figure 3.8 Effects of adrenoceptor antagonists on the spreading dilatation to adrenaline and NA in the triple-cannulated pressurized mesenteric arteries

A,B. Summarized data showing effects of α_2-adrenoceptor inhibitor yohimbine (1 μM), $\beta_{1,2}$-adrenoceptor antagonist propranolol (1 μM) and β_3-adrenoceptor antagonist SR 59230A (1 μM) on spreading dilatation responses to luminal application of adrenaline (1 μM, **A**) and NA (1 μM, **B**) in arteries preconstricted with U46619 (1 μM prazosin present).
C,D. Diagram showing effects of yohimbine, propranolol, and SR 59230A on the local (Branch 1) dilatation to adrenaline (**C**) and NA (**D**).
Results shown are the mean ± s.e.mean; $^{*}p<0.05$ vs. control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. yohimbine.

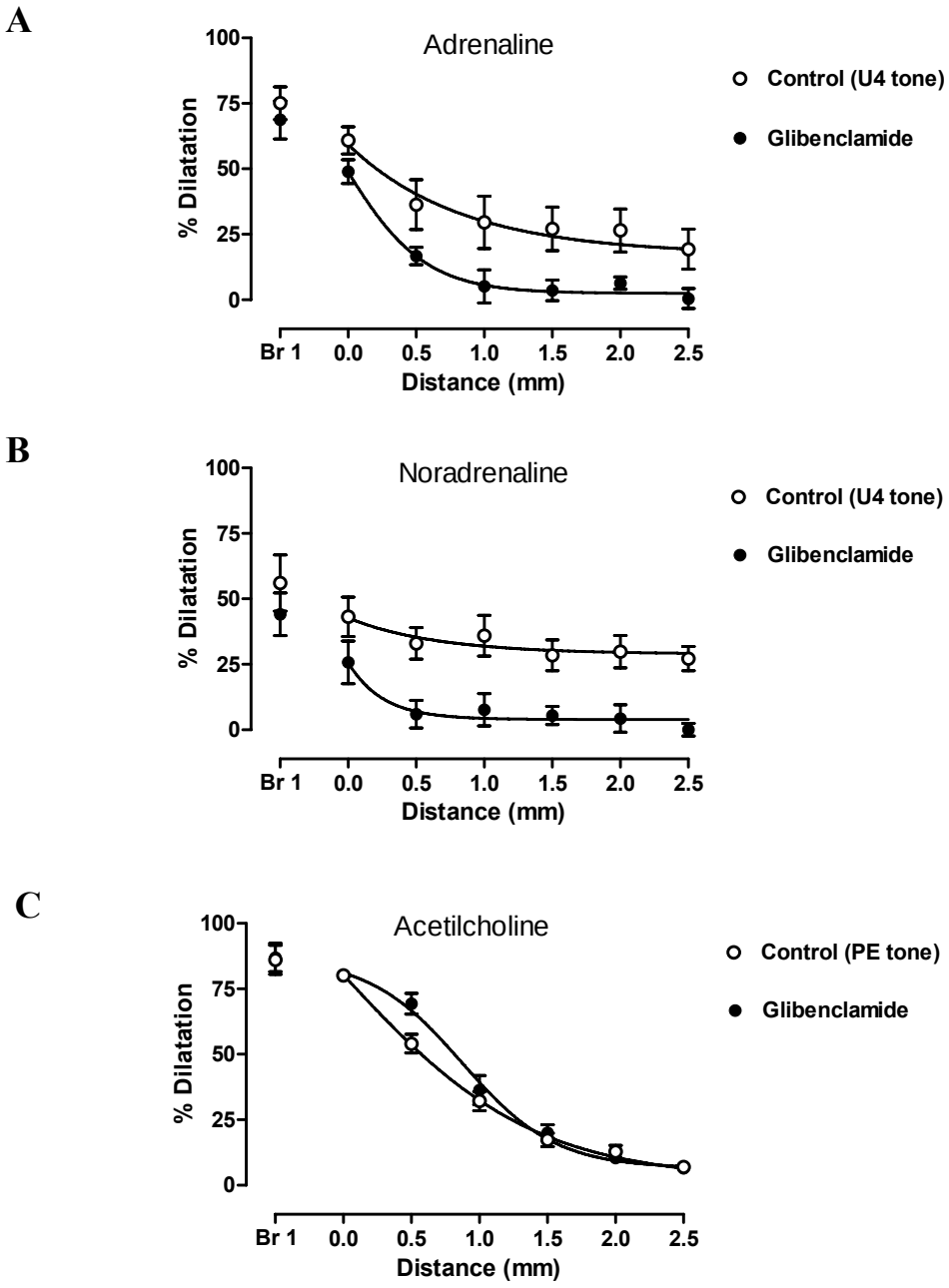


Figure 3.9 Effect of glibenclamide on the conducted dilatation in the triple-cannulated pressurized small mesenteric arteries

Summarized data showing effects of K_{ATP} channel inhibitor glibenclamide (10 μM) on the conducted dilatation responses to luminal application of adrenaline (1 μM; n = 5 - 7, **A**), NA (n = 6 - 7, 1 μM, **B**) and ACh (1 μM; n = 5 - 8, **C**). α_1-adrenoceptor inhibitor prazosin (1 μM) was present in the experiments with adrenaline and NA. Arteries were preconstricted with U46619 (U4) or phenylephrine (PE), as indicated.
Results shown are the mean ± s.e.mean.

3.3 Discussion

We were interested whether adrenergic agonists could cause a conducted vasomotor response and attempted to evaluate the pathway of this response in rat small mesenteric arteries. Our results demonstrate that β-adrenoceptor signalling can evoke conducted vasodilatation, which relies on the integrity of the endothelial cell layer. Moreover, despite a lack of functional involvement of K_{ATP} channel in the local vasomotor response, this channel was crucial for the conducted dilatation.

The sympathetic nervous system regulates arterial tone via modulation of the release of NA and adrenaline from the sympathetic nerve terminals and adrenal medulla, respectively, and their subsequent action on vascular α- and β-adrenoceptors (Guimaraes *et al.*, 2001). Constriction in rat mesenteric arteries is mediated mainly by activation of α_1-adrenoceptors (Hussain *et al.*, 2000), but not α_2-adrenoceptors (Silva *et al.*, 1996). In our experiments, adrenaline was a more potent vasoconstrictor than NA. This observation is consistent with several previous studies, in which adrenaline had a greater order of potency towards α_1-adrenoceptors than NA. This was demonstrated for the canine nasal vasculature (Berridge *et al.*, 1986) and for the human mammary artery (Bevilacqua *et al.*, 1991), but not for rat aorta (Buckner *et al.*, 1996).

Vascular tissue is known to express several subtypes of the β-adrenoceptors, and the predominant subtype in rat mesenteric arteries is still a subject of debate. According to the experiments recently performed in our lab, using selective agonists and antagonists for β-adrenoceptors, it was clearly demonstrated that the main subtype of β-adrenoceptor responsible for the hyperpolarisation of rat mesenteric arteries was the β_1-adrenoceptor, whilst the β_2-subtype played only a secondary role (Garland *et al.*, 2011). This evidence supports previously published functional studies, which revealed the contribution of both subtypes in the dilatation to β-adrenergic agonists (Zwaveling

et al., 1996). Although participation of β_3-adrenoceptors was indicated for rat mesenteric and coronary arteries (Dessy *et al.*, 2004; Figueroa *et al.*, 2009b), the β_3-adrenoceptor agonist BRL 37344 failed to evoke any significant hyperpolarization in experiments performed in our hands (Garland *et al.*, 2011). The competitive antagonism of the commonly used β_3-adrenoceptor agonist BRL 37344 with the α_1-adrenoceptors may underlie the observed vasodilatation to this agonist in preparations with PE-induced tone (Briones *et al.*, 2005), and explain the absence of hyperpolarization from resting membrane potential in rat mesenteric arteries (Garland *et al.*, 2011).

In our experiments we have used the physiological agonists, adrenaline and NA, applied in the presence of prazosin on U46619-induced tone. The dilation to adrenaline was only slightly greater then that evoked by NA, and the difference did not reach statistical significance. The dilatation to the both agonists was augmented by the α_2-adrenoceptor antagonist yohimbine. This correlates with previous studies and may be explained by an antagonizing effect of α_2-adrenoceptors on β-adrenoceptors in this tissue (Kato *et al.*, 2008), probably through the suppressive action of G_i protein on adenylyl cyclase. Indeed, the dilatation was dramatically inhibited by $\beta_{1,2}$-adrenoceptor antagonist propranolol, but, surprisingly, β_3-adrenoceptor antagonist SR 59230A further inhibited the remaining response. This, however, may be explained by the possibility of an additional inhibition of $\beta_{1,2}$-adrenoceptors by this antagonist (Hutchinson *et al.*, 2001; Manara *et al.*, 1996).

Unlike α-adrenoceptors, activation of β-adrenoceptors generally leads to a decrease in vascular tone via stimulation of adenylyl cyclase by G_s protein and subsequent enhancement of cAMP production (Guimaraes *et al.*, 2001). First, a rise in cAMP leads to hyperpolarization of the smooth muscle via activation of K_{ATP} channels (Fujii *et al.*, 1999; Nakashima *et al.*, 1995; Quinn *et al.*, 2004; Shi *et al.*, 2007; Somlyo

et al., 1970), BK_{Ca} channels (White *et al.*, 2001; Zhu *et al.*, 2006), or the delayed-rectifier voltage-gated K^+ channels (Aiello *et al.*, 1998). Second, PKA induces changes in the $[Ca^{2+}]_i$ homeostasis through inactivation of store-operated (Liu *et al.*, 2005) and L-type Ca^{2+} channels (Liu *et al.*, 1997; Orlov *et al.*, 1996; Xiong *et al.*, 1994). Finally, cAMP signalling involves stimulation of Epac (Gloerich *et al.*, 2010), which was shown to antagonise K_{ATP} channel activity (Purves *et al.*, 2009).

Recently, it has been reported for rat mesenteric artery that the β-adrenoceptor-induced hyperpolarization occurs predominantly via K_{ATP} channels. This was demonstrated by the inhibitory action of glibenclamide and a lack of the effect of the BK_{Ca} channel blocker, iberiotoxin (Garland *et al.*, 2011). The hyperpolarization was able to evoke vasodilatation through the closure of L-type Ca^{2+} channels (Ko *et al.*, 2008; Nelson *et al.*, 1995), and thus it was generally assumed that the activation of K_{ATP} channel may provide a pathway for the β-adrenoceptor-induced dilation (Quayle *et al.*, 1997). Our experiments have proved quite the opposite: inhibition of K_{ATP} channel affected neither contraction, nor dilation, caused by both adrenaline and NA. These data agree with a previous report that showed lack of the glibenclamide effect on the dilation to isoprenaline (White *et al.*, 2001) and forskolin (Omar *et al.*, 2000). This means that other mechanisms, distinct from smooth muscle hyperpolarization, e.g. modulation in Ca^{2+} homeostasis, are underlying the local vasodilatation.

It is unlikely that such a pronounced hyperpolarization, as occurs in response to β-adrenoceptor stimulation, has no physiological role. Knowing that hyperpolarisation can spread over considerable distances in rat mesenteric arteries (Takano *et al.*, 2004), we have hypothesized that although K_{ATP} channels appear not to play the main role in local dilation, they may contribute to the spreading response.

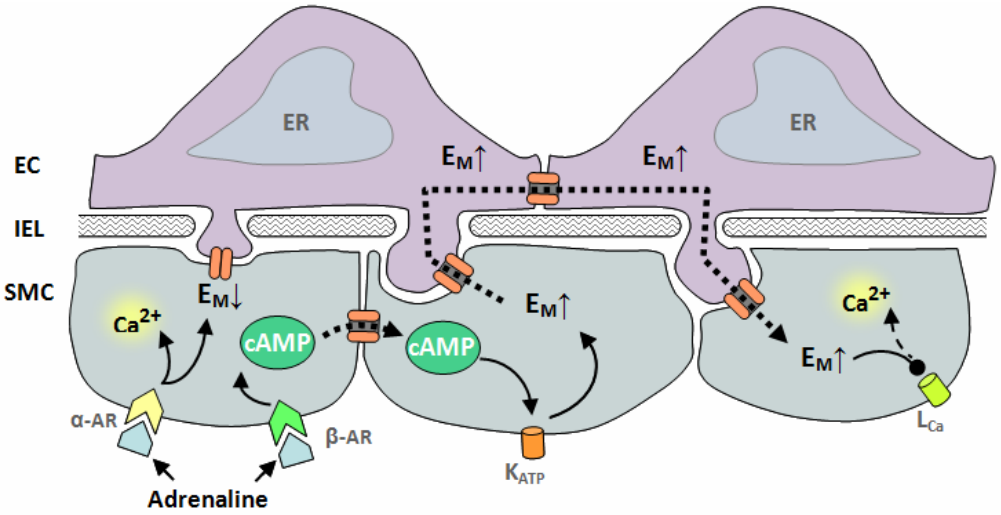


Figure 3.10 Diagram illustrating a pathway for conducted dilatation in response to adrenaline

Rise in smooth muscle cell $[Ca^{2+}]_i$ in response to stimulation of α-adrenoceptor (α-AR, yellow) leads to a local constriction, whilst stimulation of β-adrenoceptors (β-AR, green) evokes cAMP production, which could transfer to the neighbour non-stimulated cell and hyperpolarize the cell ($E_M\uparrow$) by opening of K_{ATP} channels (orange). This hyperpolarization can spread via gap junctions (brown) to adjacent endothelial cell and propagate to distant smooth muscle cells, leading to their dilatation via closure of L_{Ca} channels.

Adrenaline applied in the lumen of triple-cannulated pressurized arteries in resting conditions, evoked only a local constriction, which did not spread from the site of application. This agrees with earlier evidence that in hamster arterioles contraction to NA or KCl was constrained to the area of the application (Segal *et al.*, 1999); however, when α_1-adrenoceptors were suppressed and ~50% tone was introduced, adrenaline, in addition to local vasoconstriction, caused propagated dilatation. This conducted dilatation appeared to be a result of β-adrenoceptor signalling, since propranolol completely abolished spread. Further, in the presence of β-adrenoceptor blockers, NA/adrenaline did not evoke conducted vasoconstriction, suggesting that depolarization associated with the α-adrenoceptor-induced vasoconstriction does not spread readily to the adjacent cells in these arteries.

How the local contraction can be associated with conducted vasodilatation is not clear. It is known that smooth muscle $[Ca^{2+}]_i$ can spread to the endothelium through the myo-endothelial gap junctions, resulting in activation of endothelial NOS and K^+ channels (Dora *et al.*, 1997; Dora *et al.*, 2000; Kansui *et al.*, 2008); however, this mechanism alone is unlikely to explain the vasodilatation, as it was sensitive to propranolol or glibenclamide.

An important observation that cAMP can penetrate through the gap junctions (Ponsioen *et al.*, 2007) may help us to explain the phenomenon. Presumably, whilst the arterial segment directly exposed to adrenaline develops α_1-adrenoceptor-mediated vasoconstriction, it produces sufficient cAMP to evoke hyperpolarization in the adjacent un-stimulated segment. This hyperpolarization can spread upstream in a pattern similar to the hyperpolarization caused by the K_{ATP} channel opener levcromakalim (Takano *et al.*, 2004) (Figure 3.10). Additionally, the spread of cAMP can be enhanced by its downstream effectors, PKA and Epac, which have each been shown to enhance gap junctional communication (Duquesnes *et al.*, 2010; Popp *et al.*, 2002).

Interestingly, immediately after adrenaline was removed, the Feed branch was seen to dilate further and Branch 1 dilate beyond the resting level of tone, reaching this level in only a few minutes. This may illustrate a faster vessel recovery from the $[Ca^{2+}]_i$ rise than from the effects of elevated $[cAMP]_i$, however, the precise mechanisms responsible for this phenomenon are yet to be determined.

To elucidate the role of K_{ATP} channels in this spreading dilatation, β-adrenoceptor-mediated dilatation to the physiological relevant catecholamines, adrenaline and NA, was unmasked by the α_1-adrenoceptor inhibitor prazosin. Whilst having no effect on the ACh-mediated response, glibenclamide nearly fully suppressed the conducted dilatation to either adrenaline, NA, or isoprenaline. Denudation of the artery abolished both the

local and the conducted dilatation to ACh, whilst affecting only conducted dilatation evoked by isoprenaline. This finding corresponds to the observation that modulation of vascular tone by the endothelium may be carried by a passive spread of hyperpolarization through the myo-endothelial gap junctions, independently of changes in endothelial cell $[Ca^{2+}]_i$ (Takano *et al.*, 2004).

Finally, during our experiments we have observed an inhibitory effect of U46619 and adrenaline on ACh-mediated dilation. Whilst U46619 has been reported to inhibit SK_{Ca} channels during muscarinic receptor signalling (Crane *et al.*, 2004; Plane *et al.*, 1996), the effects of adrenaline on the ACh response will be elucidated in Chapter 5.

To summarize, the main finding of the present chapter was the demonstration that β-adrenoceptors evoked a conducted dilatator response, able to spread along the vascular wall by way of the endothelium. K_{ATP} channels appear to play a major role, contributing not to the local, initiating dilatation, but to the spreading vasodilatation, an important physiological phenomenon that has a significant impact on the regulation of vascular blood flow.

Chapter 4. Participation of the endothelium in dilatation to β-adrenoceptor stimulation

4.1 Introduction

The vascular endothelium lies at the critical interface between the blood and the blood vessel wall. It forms a semi-permeable barrier that is particularly important for controlling the passage of macromolecules and fluid between the blood and interstitial space. However, the endothelium is now known to be more than just a passive barrier against the substances dissolved in the blood plasma. It is an important organ that regulates a variety of functions including immune defence reactions, angiogenesis, thrombogenesis, and vascular smooth muscle tone.

Vascular tone is also controlled by sympathetic nervous system, through release of thcatecholamines, adrenaline and noradrenaline (NA), that target at least nine adrenoceptor subtypes, five of these, namely, $\alpha_{2A/D}$, α_{2C}, β_1, β_2, and β_3, have been identified in the vascular endothelium. Activation of these receptors either directly or acting through the release of nitric oxide (NO) may participate in the modulation of the smooth muscle tone (For review see Guimaraes *et al.*, 2001).

It is well established that the vascular endothelium has a suppressive effect on vasoconstriction (Cocks *et al.*, 1983; Dora *et al.*, 1997; Jin *et al.*, 2008; White *et al.*, 1986). The main participant in this suppression appears to be endothelial nitric oxide synthase (eNOS), since denudation of rat aorta increased sensitivity to adrenergic agonists by attenuatign NO release (Martin *et al.*, 1986). Inhibition of NOS with L-NAME also enhances the PE contractile response in rat mesenteric arteries (Ben Cheikh *et al.*, 2002; Dora *et al.*, 2000). Therefore, it was suggested that activation of eNOS in this artery is triggered by endothelial α_1-adrenoceptors (Filippi *et al.*, 2001); however, stimulation of the smooth muscle α_1-adrenoceptors was shown to have an indirect effect on the endothelium due to a Ca^{2+} signal (likely IP_3) transferring through the myo-endothelial gap junctions, leading to an increase of NO synthesis and opening of

endothelial K_{Ca} channels (Dora *et al.*, 2000; Jin *et al.*, 2008). Furthermore, freshly isolated endothelial cells PE does not increase $[Ca^{2+}]_i$ (Dora *et al.*, 2000).

On the other hand, the ability of catecholamines to evoke endothelial NO release in response to stimulation of α_2-adrenoceptors has also been well described (Guimaraes *et al.*, 2001). For example, selective α_2-adrenoceptor agonists caused concentration-dependent vasodilatation of the rat mesenteric arterial bed via NO production by the endothelium (Bockman *et al.*, 1996; Figueroa *et al.*, 2001; Pimentel *et al.*, 2007). The main subtype that is responsible for this endothelium-dependent NO-mediated relaxation is the $\alpha_{2A/D}$-adrenoceptor, which, surprisingly, did not appear to mediate this effect via the cAMP pathway (Bockman *et al.*, 1996).

It has also been suggested that endothelial β-adrenoceptors may contribute to the regulation of vascular tone; however, studies of endothelial participation in the relaxation to β-adrenoceptor agonists have given rather contradictory results. Whilst β-adrenoceptors are generally considered to stimulate eNOS, particularly in cultured endothelial cells (Hashimoto *et al.*, 2006; Kou *et al.*, 2007; Zhang *et al.*, 2006b) and conduit vessels, such as murine aorta (Akimoto *et al.*, 2002; Ferro *et al.*, 2004; Toyoshima *et al.*, 1998) or carotid artery (MacDonald *et al.*, 1999), removal of endothelium or eNOS inhibition affected the vasodilatation in rat mesenteric arteries in only a fraction of cases (Figueroa *et al.*, 2009b; Graves *et al.*, 1993; Kozlowska *et al.*, 2003). For example, in perfused mesenteric artery, stimulation of adrenoceptors with adrenaline was shown to evoke transient NO release and eNOS phosphorylation at serine 1177. Nevertheless, a selective β-adrenoceptor agonist, isoprenaline, failed to lead to eNOS phosphorylation (Figueroa *et al.*, 2009b). Others have reported no effect of eNOS inhibition on the dilatation responses to isoprenaline (Blankesteijn *et al.*, 1993; Briones *et al.*, 2005) or forskolin (Simonsen *et al.*, 1999). Moreover, endothelium

denudation or inhibition of eNOS was even shown to augment β-adrenoceptor-mediated relaxation in this preparation (Iwatani *et al.*, 2008).

In the previous chapter (Chapter 3) we demonstrated that the endothelium is crucial for the conducted dilatation in response to β-adrenoceptor stimulation. It is not clear, however, whether the endothelium contributes to the local dilatation in response to β-adrenoceptor signalling in rat small mesenteric arteries. Therefore we decided to examine the role of endothelium and endothelium-derived relaxing factors (EDRFs) in the local dilatation to adrenaline and NA by means of inhibition of EDRF pathways, endothelium removal and comparison of luminal and abluminal applications of the adrenergic agonists.

4.2 Methods

4.2.1 Pressure myography

In order to enable selective application of studied agonists on endothelial or smooth muscle cell layers of the artery, the pressure myograph system was used in these experiments. For this experiments, segments of arteries free of side branches were cut to give a final length of >2 mm, cannulated with two glass pipettes in a bath of the temperature-regulated chamber (10 mL, 120CP, Danish Myo Technology, Denmark) and pressurized to 70 mm Hg, as described in Section 2.2. A syringe pump system (BeeHive syringe pump system, Bioanalytical systems, USA) was connected to one end of the artery, whilst the other end was attached to the gravity-fed pressure system allied to the open-ended reservoir (5 ml). To obtain denuded arteries, pressure was decreased and an air bubble (~2.5 mm^3) was perfused through the artery. The lumen was then washed and the pressure was re-introduced. After 20 minutes of equilibration period, removal of the endothelium was assessed by loss of the dilatation to ACh (0.1 -10 μM).

4.2.2 Luminal and abluminal cumulative concentration-response curves

The studied agonists were added directly to the bath (abluminally) or infused via lumen, as described in Section 2.2.1 (Figure 4.1). To obtain abluminal concentration-response curves, adrenaline or NA were cumulatively applied in bath with time increments of not less then two minutes, to allow the response to reach a plateau (Figure 4.2A). Due to the pipette dead volume, the minimal waiting time between the concentrations for luminal infusions was three minutes (Figure 4.2B). Dilatation concentration-response curves were obtained following submaximal (70-80% of maximal tone) preconstriction with PE or, in the cases when α-adrenergic inhibitors were applied, a stable TP receptor agonist U46619.

4.2.3 Data analysis

Data were analyzed as described previously (see Section 2.5).

4.2.4 Drugs and solutions

See section 2.6 for the preparation details.

MOPS buffered solution was used throughout in these experiments. Solutions containing L-NAME (100 μM) had their pH additionally adjusted to 7.4±0.02.

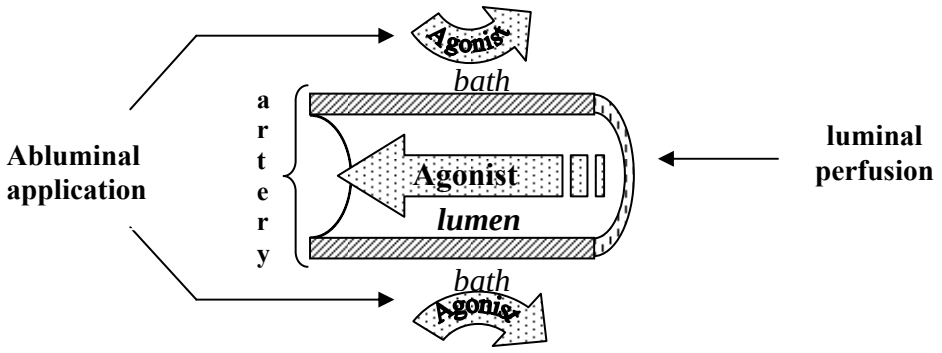
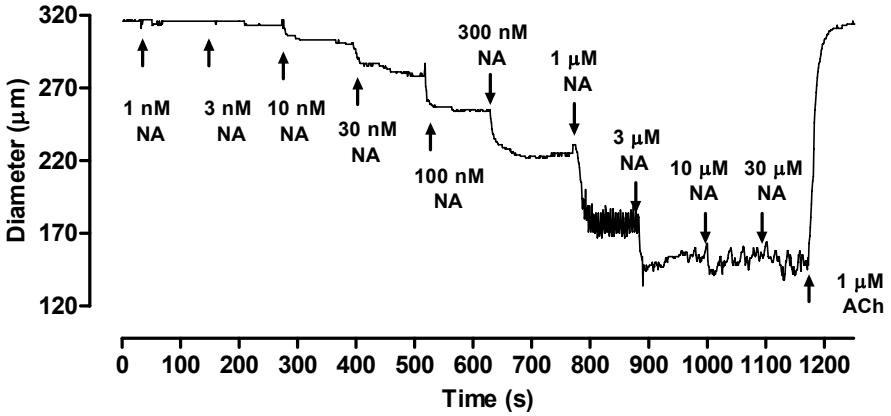


Figure 4.1 Diagram illustrating cross-section of the vessel during luminal perfusion or abluminal application of studied agonists

During experiments drugs were either applied directly in bath (abluminally), or infused via the lumen by means of syringe pumps (BeeHive syringe pump system). This technique allowed the selective application of studied compounds at smooth muscle or endothelial cell sides of the artery.

A



B

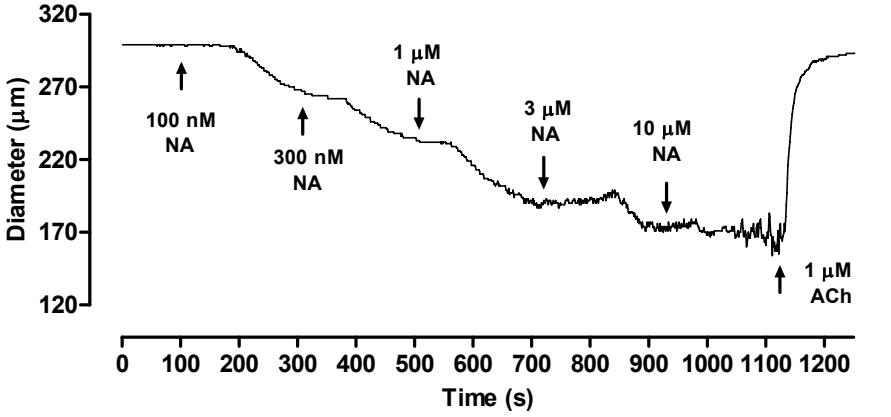


Figure 4.2 Constriction of pressurized small mesenteric arteries to NA

A. Original trace showing concentration-dependent constriction of rat mesenteric arteries to abluminal application of NA.
B. Original trace illustrating concentration-dependent constriction NA perfused through the lumen.
Arrows indicate the timing of NA application. Note that the dead space volume of the pipette is causing a delay in the response to luminally perfused NA. The muscarinic cholinergic receptor agonist ACh (1 µM) was applied at the end of each trace to examine endothelial cell viability.

4.3 Results

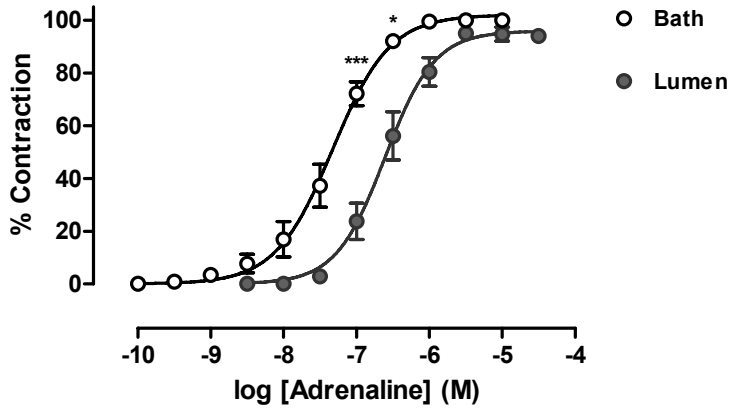
4.3.1 Comparison of the constriction responses to luminal and abluminal application of adrenergic agonists; effect of NOS inhibition

To establish the involvement of the endothelium in the dilatation to adrenergic agonists, in this section we have examined whether luminal perfusion of adrenaline and NA results in reduced contractility due to release of EDRF.

First, cumulative concentration-response curves to these agonists were obtained during luminal perfusion and compared to those obtained by abluminal application. The concentration-response curves obtained luminally were significantly shifted to the right for both adrenaline (from pEC_{50} = 7.32 ± 0.06, n = 10, to pEC_{50} = 6.6 ± 0.07, n = 6, p<0.05; Figure 4.3A) and NA (from pEC_{50} = 6.43 ± 0.04, n = 9, to pEC_{50} = 5.89 ± 0.05, n = 11, p<0.05; Figure 4.3B). Adrenaline was a more potent agonist than NA when applied by both methods (p<0.05), as observed in the wire myograph experiments (See Chapter 3, Figure 3.3A).

Since there was a shift between luminal and abluminal applications of the agonists, we have examined whether this shift was mediated by EDRF. For this, we have used NOS inhibitor L-NAME (100 μM) and compared its effect on luminal and abluminal concentration-response curves. Inhibition of eNOS augmented both adrenaline and NA abluminal responses (from pEC_{50} = 7.32 ±0.06, n = 10, to pEC_{50} = 7.74±0.04, n = 6, for adrenaline (p<0.05); and from pEC_{50} = 6.43±0.04, n = 9, to pEC_{50} = 6.79+0.05, n = 7, for NA (p<0.05)); however, constriction to luminal application of these agonists was not affected by L-NAME (P>0.05; Figure 4.4 and 4.5), indicating that the reduction in the contractile response to lumenally perfused agonists was not mediated by eNOS signalling.

A



B

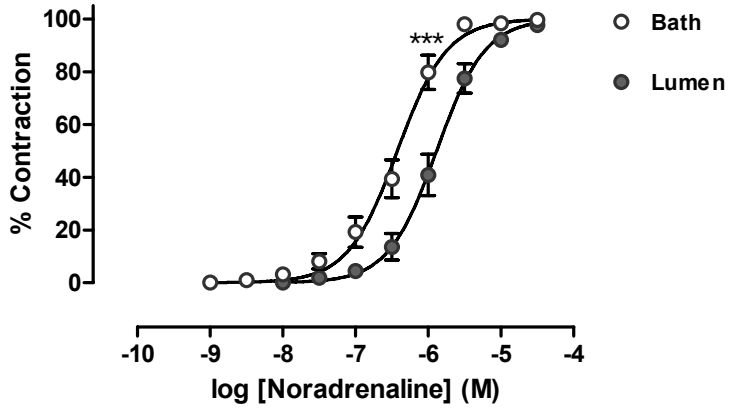
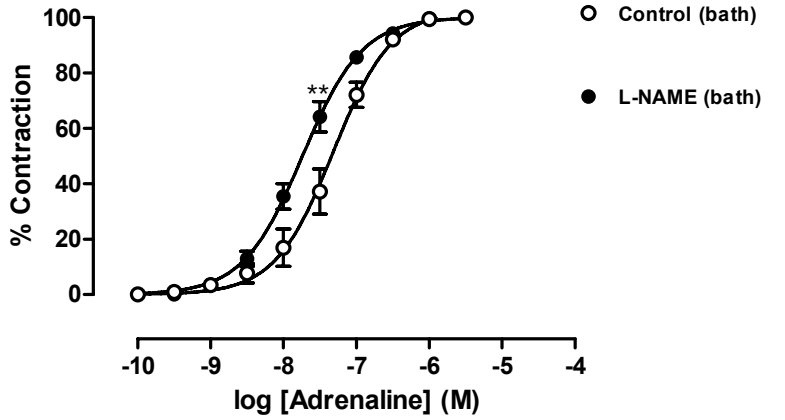


Figure 4.3 Concentration-response curves of constriction to adrenaline and NA applied luminally or abluminally in pressurized small mesenteric arteries

A. Summarized data demonstrating contraction to rising concentrations of adrenaline, applied abluminally (in bath; n = 10) or perfused luminally (lumen; n = 6, $p<0.05$).
B. Summarized data demonstrating constriction to rising concentrations of NA applied abluminally (n = 9) or luminally (n = 11, $p<0.05$).

Results shown are the mean ± s.e.mean, *$p<0.05$, ***$p<0.001$ lumen vs. bath.

A



B

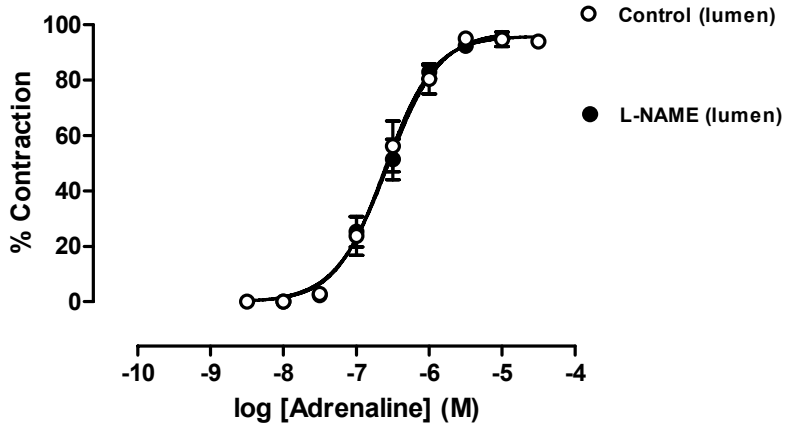
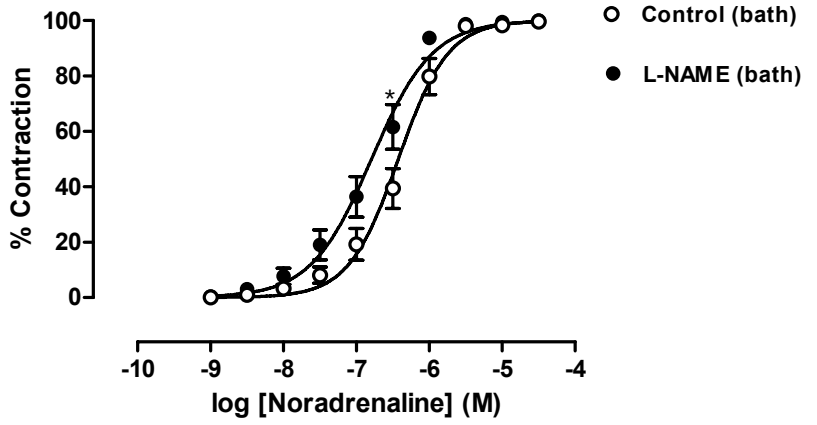


Figure 4.4 The effect of NOS inhibition on the constriction to adrenaline in pressurized small mesenteric arteries

A. Concentration-response curves demonstrate augmented constriction to adrenaline applied abluminally after the inhibition of NOS with L-NAME (100 μM, $n = 6$ - 10, $p<0.05$).
B. Concentration-response curve to luminal perfusion of adrenaline was not modified by L-NAME, ($n = 6$, $p>0.05$).

Results shown are the mean ± s.e.mean, *$p<0.05$ vs. control.

A



B

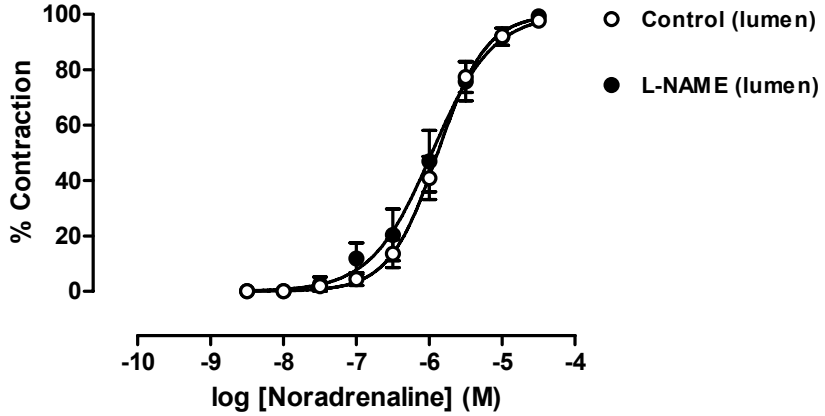


Figure 4.5 The effect of NOS inhibition on the constriction to NA in pressurized small mesenteric arteries

A. Concentration-response curves demonstrate augmented constriction to NA applied abluminally after inhibition of NOS with L-NAME (100 μM, n = 7-9, p<0.05).
B. Concentration-response curve to luminal perfusion of NA was not modified by L-NAME (n = 6 - 11, p>0.05).

Results shown are the mean ± s.e.mean, *p<0.05, vs. control.

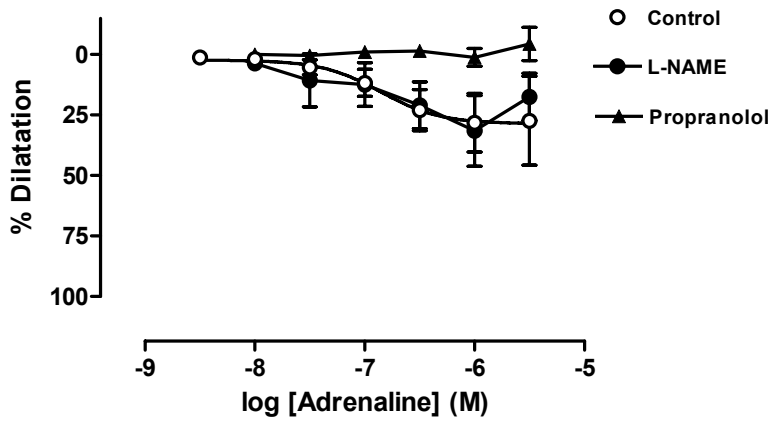
4.3.2 Dilatation of arteries preconstricted with PE to the luminal application of adrenergic agonists

To establish whether application of adrenergic agonists via lumen can evoke endothelium-dependent dilatation, rat small mesenteric arteries were submaximally preconstricted with α_1-adrenoceptor agonist PE, and then NA and adrenaline were luminally perfused. Both NA and adrenaline relaxed arteries to 34.7 ± 10% (n = 9) and 28.2 ± 12% (n = 7), respectively (Figure 4.6), whilst luminal perfusion of MOPS buffer by itself had no significant effect, as it has been shown previously (Rodriguez-Rodriguez *et al.*, 2009). To elucidate whether EDRFs participated in the adrenaline-evoked dilatation, the NOS inhibitor L-NAME was applied, and it did not affect the dilatation (P>0.05). Application of propranolol (1 μM) alone blocked the dilatation, supporting the suggestion of β-adrenoceptors involvement (Figure 4.6).

4.3.3 Comparison of the dilatation to the luminal and abluminal application of adrenergic agonists of arteries preconstricted with U46619

In order to unmask the relaxation to adrenergic agonists, α_1-adrenoceptor antagonist prazosin (1 μM) was used and arteries were submaximally preconstricted with thromboxane mimetic U46619. Prazosin revealed a nearly maximal dilatation to the abluminally applied adrenaline (E_{max} = 86.1 ±6.7%, n = 4) and NA (E_{max} = 90.20 ± 6.4%, n = 6); however, luminal perfusion resulted in a rightward shift of the concentration-response curves for adrenaline (from pEC_{50} = 6.44 ± 0.2, n = 4, to pEC_{50} = 5.8 ± 0.3, n = 5; Figure 4.7A) and NA (from pEC_{50} = 6.22 ± 0.06, n = 8, to pEC_{50} = 5.63 ± 0.02, n = 6, p<0.05; Figure 4.7B), but not for a lipophilic adenylyl cyclase activator forskolin (from pEC_{50} = 6.33 ± 0.04, n =11, to pEC_{50} = 6.36 ± 0.03, n = 5; Figure 4.7C).

A



B

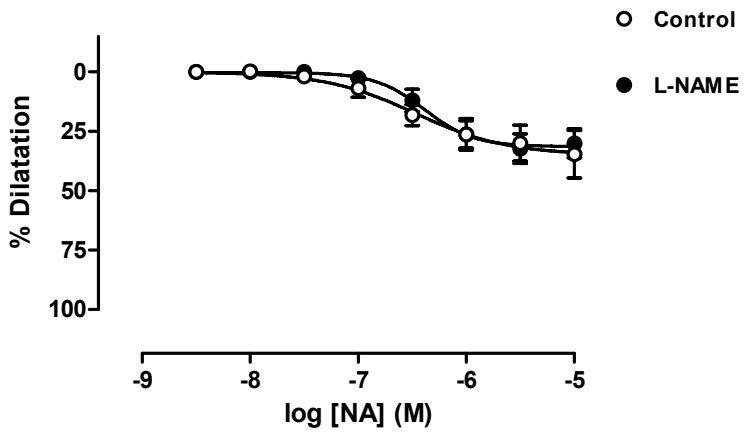


Figure 4.6 The effect of luminal perfusion of adrenaline and NA in pressurized small mesenteric arteries preconstricted with PE

A. Concentration-response curves represent effects of NOS inhibitor L-NAME (100 μM; *n* = 5) and β-adrenoceptor antagonist propranolol (1 μM; *n* = 3) on the dilatation to luminal applied adrenaline (*n* = 9).
B. Dilatation to luminally perfused noradrenaline (NA) was also unaffected by NOS inhibition (*n* = 4 - 9).

Results shown are the mean ± s.e.mean.

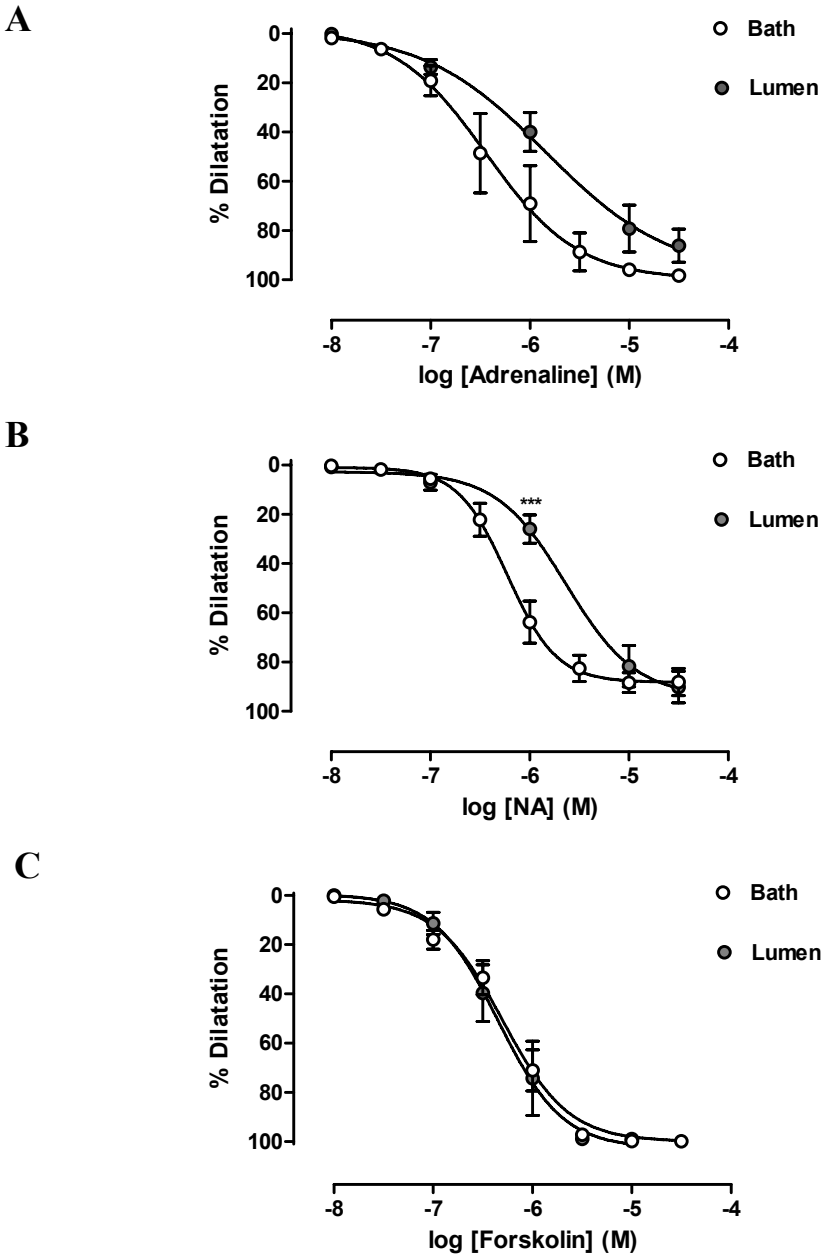


Figure 4.7 Comparison of the dilatation concentration-response curves obtained to luminal vs. abluminal application of agonists in pressurized small mesenteric arteries

A. In the presence of the α_1-adrenoceptor antagonist prazosin (1 μM), arteries preconstricted with U46619 dilated to abluminal application of adrenaline with a greater potency than to luminal perfusion (n = 4 - 5). **B.** Arteries preconstricted with U46619 in the presence of prazosin also displayed an enhanced dilatation to abluminal applications of NA compared to luminal perfusion (n = 6 - 8, p>0.05). **C.** Concentration-response curves to luminal perfusion of forskolin were not different to abluminal application (PE tone; n = 5 - 11, p>0.05).
Results shown are the mean ± s.e.mean; ***p<0.001 vs. bath.

4.3.4. The role of the endothelium in the dilatation to luminally perfused adrenergic agonists

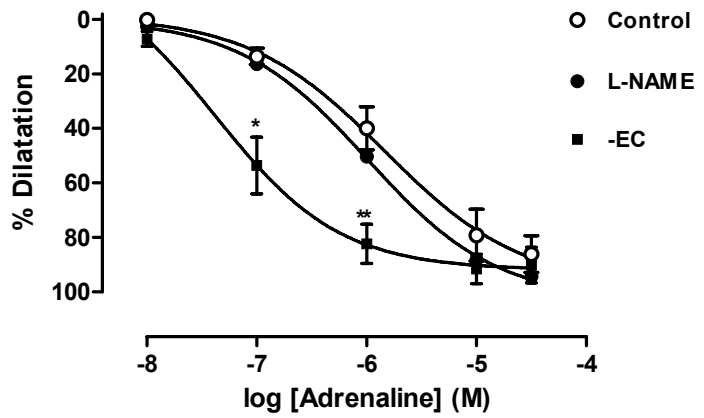
These experiments were designed to reveal a possible role of the endothelium in the dilatation to luminally applied adrenaline or NA. In the presence of prazosin, both luminally applied catecholamines evoked concentration-dependent dilatation of U46619– preconstricted arteries, which was insensitive to the inhibition of eNOS with L-NAME (pEC_{50} = 5.99 ± 0.39, n = 3, p>0.05, for adrenaline, and pEC_{50} = 5.71 ± 0.17, n = 6, p>0.05, for NA). Denudation of the artery markedly augmented the dilatation, with pEC_{50} values reaching 7.37 ± 0.89 for adrenaline (n = 3) and 6.94 ± 0.09 for NA (n = 6) (Figure 4.8).

4.3.5. The role of the endothelium in the relaxation to abluminally applied adrenergic agonists

Arteries preconstricted with U46619 in the presence of prazosin submaximally dilated to abluminally applied adrenaline (E_{max} = 98.3 ± 0.8%, n = 4) and NA (E_{max} = 88.1 ± 5.5%, n = 6). This relaxation was insensitive to L-NAME (pEC_{50} = 6.44 ± 0.04, n = 4, p>0.05, for adrenaline, and pEC_{50} = 6.22 ± 0.06, n = 9, p>0.05, for NA; Figure 4.9A.B). Additional inhibition of IK_{Ca} and SK_{Ca} with TRAM-34 (1 μM) and apamin (50 nM) resulted in a moderate leftward shift of NA concentration-response curve (pEC_{50} = 6.51 ± 0.04, n = 4, p>0.05; Figure 4.9B). Denudation of the artery has slightly augmented the potency to NA (pEC_{50} = 6.38 ± 0.05, n = 8, p>0.05; Figure 4.9B).

When a selective β-adrenoceptor agonist, isoprenaline, was used, L-NAME also failed to affect the dilatation of the PE-preconstricted arteries (from pEC_{50} = 7.07 ± 0.09, to pEC_{50} = 7.16 ± 0.09, n = 5; p>0.05; Figure 4.9B). These data indicate the lack of participation of EDRF in the relaxation to β-adrenoceptor stimulation.

A



B

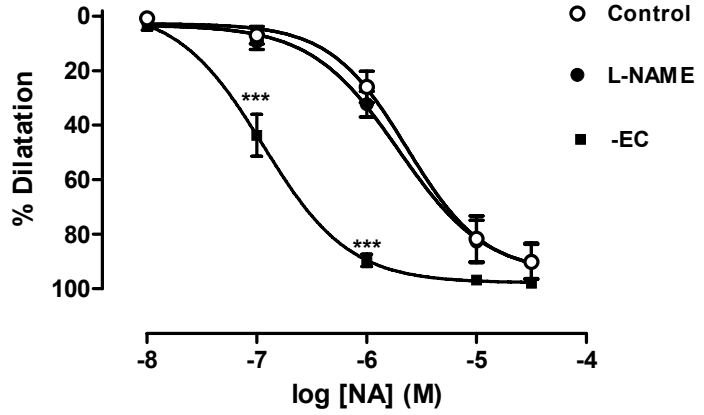


Figure 4.8 Involvement of the endothelium and eNOS in the dilatation to luminally perfused adrenaline and NA in pressurized small mesenteric arteries

A. Concentration-response curve demonstrating the effect of NOS inhibitor L-NAME (100 μM) or endothelium denudation (-EC) on the concentration-dependent relaxation to lumenally applied adrenaline (n = 3 - 5).
B. Effect of L-NAME or endothelium denudation on concentration-dependent relaxation to lumenally applied noradrenaline (NA; n = 5 - 6).
Arteries were preconstricted with thromboxane mimetic U46619 in the presence of α-adrenoceptor antagonist (prazosin or phentolamine, 1 μM)
Results shown are the mean ± s.e.mean, *p<0.05, **p<0.01, ***p<0.001 vs. control.

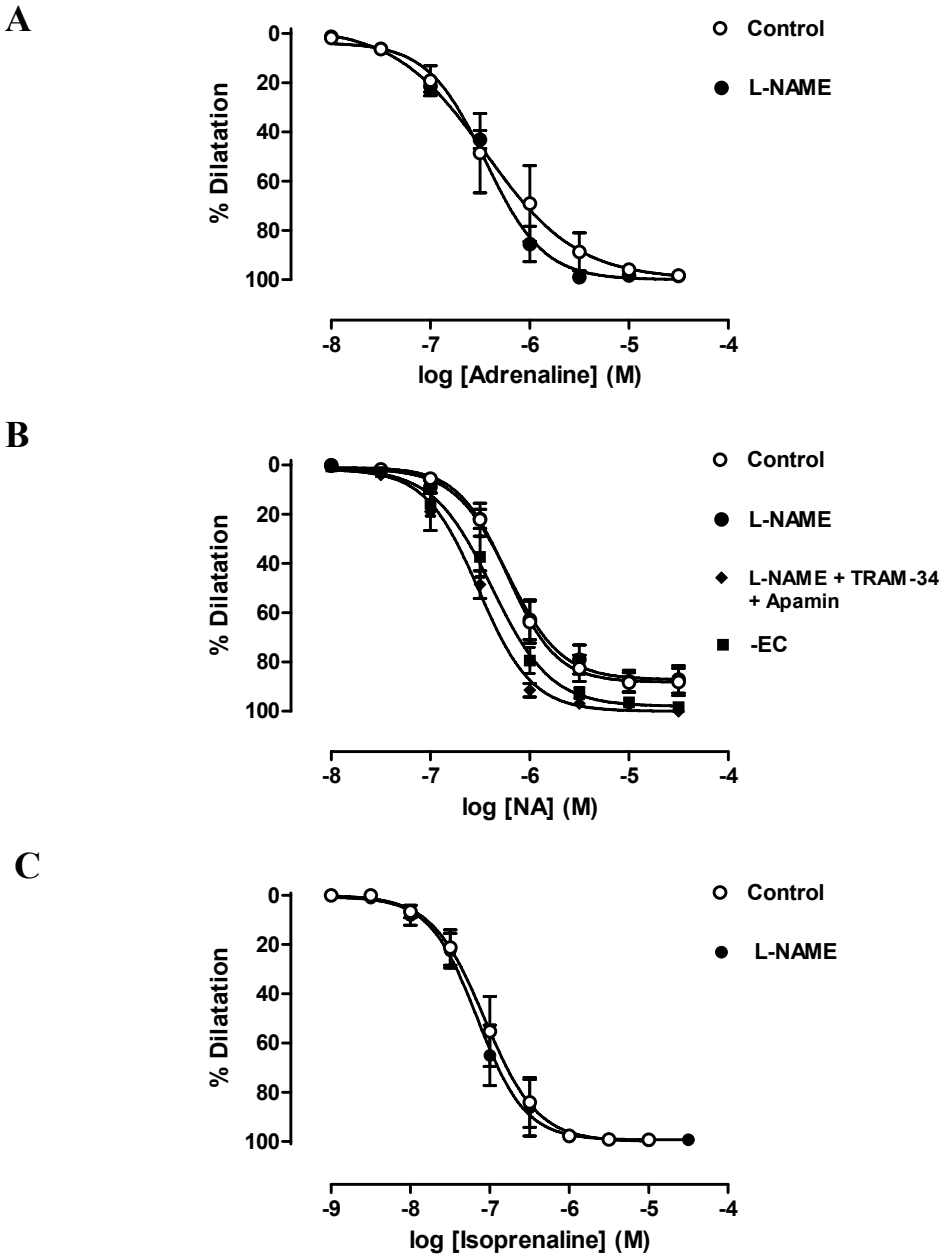


Figure 4.9 Involvement of EDRFs in the dilatation of pressurized small mesenteric arteries to abluminal application of adrenergic agonists

A. Concentration-response curves showing the effect of L-NAME (100 μM) on the dilatation to adrenaline, applied in bath (n = 4, p>0.05). **B.** Effects of endothelium denudation (-EC), L-NAME, TRAM-34 (1 μM) and apamin (50 nM) on the dilatation to NA (n = 3-9, p>0.05). Arteries were preconstricted with U46619 in the presence of the α_1-adrenoceptor antagonist prazosin (1 μM; A,B). **C.** Effect of L-NAME on the dilatation of PE-preconstricted arteries to β-adrenoceptor agonist isoprenaline (n = 8, p>0.05).
Results shown are the mean ± s.e.mean.

4.4 Discussion

The vascular endothelium lining blood vessels is known to be a semi-permeable barrier that regulates the flux of molecules between the blood and surrounding tissue. The permeability of transferred substances depends on their molecular radius (Mehta *et al.*, 2006) as well as solubility in lipids (Lew *et al.*, 1989). Endothelial cells are therefore the first to be exposed to luminally applied molecules and their action on smooth muscle cells will depend on the rate of diffusion through the endothelium.

In accordance with this, we have tested the hypothesis that luminally applied adrenergic agonists may predominantly stimulate endothelial adrenoceptors and lead to release of endothelium-derived relaxing factors. Our experiments demonstrated a rightward shift of the constriction concentration-response curve for the luminally perfused catecholamines in comparison to abluminal application, but this shift was not due to release of endothelium-derived relaxing factors, but due to a restricted permeability of the endothelial layer to the hydrophilic molecules that may increase dilution of the agonist in smooth muscle tissue. This assumption was supported by a similar rightward shift of the dilatation concentration-response curve to the luminally infused adrenaline and NA that was reversed by removal of the endothelium. Interestingly, a lipophilic molecule forskolin, which also activates adenylyl cyclase, did not display such rightward shift of the luminally obtained concentration-response curve. This agrees with the observation that the endothelial barrier in small arteries is less permeable to water soluble molecules than to lipophilic ones (Lew *et al.*, 1989).

Stimulation of eNOS in rat mesenteric artery has been suggested to be triggered by endothelial α_1-adrenoceptors (Filippi *et al.*, 2001) α_2-adrenoceptors (Bockman *et al.*, 1996; Figueroa *et al.*, 2001; Pimentel *et al.*, 2007; Rascado *et al.*, 2005), partially β_1-adrenoceptors (Graves *et al.*, 1993; Huang *et al.*, 1998) and all three subtypes of β-

adrenoceptors (Figueroa *et al.*, 2009b). To clarify the role of eNOS in the dilatation, we have examined the effect of NOS inhibition on both constriction and dilatation responses to tested catecholamines. During constriction to abluminal application of adrenaline and NA, inhibition of eNOS resulted in a leftward shift of concentration-response curves. A similar effect was already described for both, conduit vessels, such as rat aorta (Martin *et al.*, 1986), and resistance vessels, such as rat mesenteric artery (Dora *et al.*, 2000), and can be explained by basal activity of eNOS (Martin *et al.*, 1986; Simonsen *et al.*, 1999) as well as by an indirect effect of smooth muscle Ca^{2+} signalling through myo-endothelial gap junctions (Dora *et al.*, 2000; Kansui *et al.*, 2008). Intriguingly, the shift was present only in case of abluminal applications of catecholamines, whereas luminal concentration-response curves remained the same after NOS inhibition, as if the NO pathway was already inactivated. This observation led us to further experiments, where effects of β-adrenoceptor signalling on endothelium-derived relaxation were evaluated (see the results in Chapter 5).

In contrast to constriction, the dilatation to both luminal and abluminal application of adrenaline and NA was not affected by inhibition of eNOS. Since there was evidence of EDHF-type responses to β-adrenergic agonists (Dessy *et al.*, 2004; Huang *et al.*, 1998), we have inhibited EDHF by blocking SK_{Ca} and IK_{Ca} channels; however this, as well as endothelial denudation, resulted rather in slight augmentation of the response. Our data therefore support the assumption that the endothelium in rat mesenteric arteries regulates and maintains vascular tone via counteracting not only vasoconstriction, but also vasodilatation, possibly at least in part by release of endothelium-derived contracting factors (Iwatani *et al.*, 2008) (this will be discussed in the Chapter 6 in detail). Additionally, the inhibition of the vasodilatation following endothelial disruption seen in several studies (Figueroa *et al.*, 2009b; Graves *et al.*, 1993) may be

explained by a non-specific constitutive influence of the endothelium on the smooth muscle rather than the action of endothelial β-adrenoceptors (Briones *et al.*, 2005).

Several studies demonstrated that β_3 is the adrenoceptor subtype that was predominantly involved in activation of eNOS. This was shown for cultured endothelial cells (Kou *et al.*, 2007), rat aorta (Trochu *et al.*, 1999), carotid (MacDonald *et al.*, 1999), and mesenteric arteries (Figueroa *et al.*, 2009b). In these studies, the β_3-adrenoceptor agonist BRL 37344 or antagonist SR 59230A were used to establish an involvement of the β_3-adrenoceptor in the release of NO; however, other authors strongly argue against β_3-adrenoceptor-mediated dilatation responses in rat mesenteric artery and aorta and point out that both compounds are antagonists of α_1-adrenoceptors (Brahmadevara *et al.*, 2003; Briones *et al.*, 2005). Notably, in rat mesenteric arteries, β-adrenoceptor agonists isoprenaline and BRL 37344 were substantially less effective in inducing NO release, than the subtype non-selective agonist adrenaline (Figueroa *et al.*, 2009b). Therefore it could be suggested that activation of α_1-adrenoceptors located on smooth muscle cells evokes rise in $[Ca^{2+}]_i$ that can be transferred to the endothelium, leading to activation of eNOS (Dora *et al.*, 2000; Kansui *et al.*, 2008). An additional mechanism of NO release may provide neuronal NOS, which also participates in responses to β-adrenoceptor stimulation in this tissue (Blanco-Rivero *et al.*, 2006a).

On the other hand, inhibition of NOS was shown to cause down-regulation of β-adrenoceptors in anesthetized rats (Whalen *et al.*, 2006), thus, it seems possible that L-NAME in some cases attenuated the response to β-adrenergic agonists due to enhanced desensitization rather than due to disruption of the dilatation pathway, provided by the endothelium.

Importantly, the non-selective adrenoceptor agonist NA, which has been used as a contractile agent in many experiments, was shown to promote NO release in

isoprenaline-induced relaxation via stimulation of α_2-adrenoceptors in endothelial cells (Rascado *et al.*, 2005). In our experiments though, we have used a selective α_1-adrenoceptor agonist PE, which to circumvent the pathway described above (Rascado *et al.*, 2005).

Endothelial denudation was shown to amplify α_2-adrenoceptor-mediated contraction in rat aorta (Martin *et al.*, 1986) and mesenteric artery (Blanco-Rivero *et al.*, 2006b; White *et al.*, 1986); it was also reported that eNOS may be involved in the dilatation in response to α_2-adrenoceptor agonists (Bockman *et al.*, 1996; Figueroa *et al.*, 2001; Pimentel *et al.*, 2007; Rascado *et al.*, 2005). On the other hand, the dilatation to the commonly used α_2-adrenergic agonist clonidine may signal through the activation of α_1-adrenoceptor (Silva *et al.*, 1996) and lead to a rise in endothelial cell $[Ca^{2+}]_i$. Besides that, in our experiments propranolol alone attenuated, whereas yohimbine did not suppress, but enhanced the dilatation to adrenaline and NA (see Chapter 3). This observation supports the observation that α_2-adrenoceptors seem to exert rather an inhibitory effect on β-adrenoceptor signalling (Kato *et al.*, 2008), leading to reduced dilatation to physiological β-adrenergic agonists.

A lack of selective β-adrenoceptor antibodies was successfully substituted by the β-adrenoceptor fluorescent ligand BODIPY, which confirmed the presence of β-adrenoceptors in the adventitia, media and intima of rat small mesenteric arteries (Briones et al., 2005). The fact that β-adrenoceptors were not exclusively localized in the smooth muscle cells, but also in the endothelium, allows us to suggest that this receptor may play as yet unidentified role. Endothelial cell β-adrenoceptors may participate in regulation of the intima permeability through their downstream effectors PKA and Epac (Birukova *et al.*, 2009), or enhance gap junctional coupling and thereby promote conducted vasomotor response (please see Chapter 6) (Duquesnes *et al.*, 2010;

Popp *et al.*, 2002). Our preliminary data also demonstrate an increase in the frequency and extent of $[Ca^{2+}]_i$ events in endothelial cells in response to isoprenaline (see Chapter 7). Whether this effect is attributed to signalling of smooth muscle cells (SMCs) or endothelial cells is the subject of ongoing experiments.

To summarise, in this chapter we have examined the contribution of the endothelium in the dilatation of rat small resistance mesenteric arteries to physiological adrenergic agonists, adrenaline and NA. The results of this study demonstrate that the endothelium does not seem to play a direct role in the dilatation to β-adrenoceptor stimulation. It rather creates a semi-permeable barrier, which prevents luminal catecholamines from acting on the smooth muscle cell receptors. However, the endothelium is crucial for coordinating the relaxation and as shown in the previous chapter, enabling propagated vasodilatation to β-adrenoceptor agonists.

Chapter 5. Effects of β-adrenoceptor stimulation on the endothelium-dependent dilatation

5.1 Introduction

The endothelium modulates vascular tone by means of release of vasoactive factors as well as by providing a low-resistance pathway for hyperpolarizing currents (Dora, 2010; Takano *et al.*, 2004). Dysfunction of this endocrine gland is known to be implicated in the development of many cardiovascular diseases, one of which is hypertension (Dharmashankar *et al.*, 2010; Feletou *et al.*, 2010b).

Raised levels of adrenaline and/or NA in blood plasma are associated with cardiovascular pathogenesis (Borkowski *et al.*, 1992; Brown *et al.*, 1981; Floras, 1992; Gayen *et al.*, 2010; Goldstein, 1983; Grassi *et al.*, 2010; Higashi *et al.*, 2002; Majewski *et al.*, 1981; Tung *et al.*, 1981). One of the predictors for a poorer cardiovascular risk status is also an increased sympathoadrenal reactivity to stress (Chida *et al.*, 2010; Flaa *et al.*, 2008; Lambert *et al.*, 2010), which results in elevated plasma adrenaline concentration (Brown *et al.*, 1981; Floras, 1992). Adrenaline may activate adrenoceptors expressed on the intima, media and adventitia. Generally, α-adrenoceptors are mainly involved in vasoconstriction, whilst β-adrenoceptors evoke vasodilatation in mesenteric arteries. β-adrenoceptors stimulate adenylyl cyclase to elevate cellular cAMP levels, leading to relaxation of the smooth muscle (see Section 1.2.5 for details). Despite the evidence that β-adrenoceptors are expressed in the endothelium and can induce endothelium-dependent relaxation in larger vessels (Ferro *et al.*, 2004; Figueroa *et al.*, 2009b; Nishina *et al.*, 1999), our previous investigations failed to prove involvement of the endothelium in the local dilatation of rat small mesenteric arteries to β-adrenergic agonists (see Chapter 4).

Activation of healthy endothelium, for example in response to ACh or ATP, is associated with a rise in endothelial cell $[Ca^{2+}]_i$ (McSherry *et al.*, 2005; Mumtaz *et al.*, 2010; Rodriguez-Rodriguez *et al.*, 2009; Takano *et al.*, 2004), which stimulates

endothelial nitric oxide synthase (eNOS) and cyclooxygenase (COX), leading to release of nitric oxide (NO) and prostanoids. It can also stimulate the intermediate and small conductance Ca^{2+}-sensitive K^+ channels (IK_{Ca} and SK_{Ca}, respectively); activation of these channels causes an efflux of K^+ and hyperpolarization of the endothelium (Chen *et al.*, 1997; Dora *et al.*, 2008; Edwards *et al.*, 1998; Zygmunt *et al.*, 1996). This hyperpolarisation is a key event for the formation of NO- and prostanoid-independent endothelium derived hyperpolarization (EDH) of vascular smooth muscle cells (Brahler *et al.*, 2009; Garland *et al.*, 1992b; Taylor *et al.*, 1988). EDH can be transferred from endothelial cells to smooth muscle cells by two main mechanisms: stimulation of smooth muscle K_{ir} channels and the Na^+/K^+-ATPase with K^+ released from endothelial IK_{Ca} channels (Dora *et al.*, 2008; Doughty *et al.*, 1999; Edwards *et al.*, 1998), and/or conduct of the hyperpolarization derived from SK_{Ca} channels via myo-endothelial gap junctions (Coleman *et al.*, 2002; Dora *et al.*, 2008; Dora *et al.*, 1999; Dora *et al.*, 2003a; Figueroa *et al.*, 2009a). Regardless of the mechanism, hyperpolarization of SMC closes L_{Ca} channels and results in vasodilatation. The vasodilatation can be spatially magnified by the ability of EDH to spread both longitudinally and radially through the myo-endothelial gap junctions (Dora, 2001; Dora *et al.*, 2003b; Segal *et al.*, 1989; Takano *et al.*, 2004; Yamamoto *et al.*, 2001). Signalling pathways triggered by many stimuli, such as activation of adrenoceptors, involve protein kinases A and C, which can modulate the conducted vasodilatation (Bao *et al.*, 2007; Haug *et al.*, 2003; Heyman *et al.*, 2009; Inoguchi *et al.*, 1995; Popp *et al.*, 2002) (see Section 1.4 for details).

Arteries from hypertensive humans and rats were shown to have an impaired endothelial function and eNOS activity (Higashi *et al.*, 2002; Li *et al.*, 2007; Linder *et al.*, 1990; Wang *et al.*, 2010), supporting a suggestion that deficiency of NO is implicated in the pathogenesis of cardiovascular disease (Feletou *et al.*, 2010b;

Kedziora-Kornatowska *et al.*, 2006; Kleinbongard *et al.*, 2006; Schulz *et al.*, 2000; Wang *et al.*, 2010). There is also a clear link between impairment of the EDH pathway and hypertension (Brahler *et al.*, 2009; Feletou *et al.*, 2010b; Figueroa *et al.*, 2009a; Grgic *et al.*, 2009); however, despite extensive studies, the precise mechanism linking an increased sympathoadrenal reactivity and cardiovascular pathogenesis is still to be determined (Lambert *et al.*, 2010).

Recently, it was shown that activation of adenylyl cyclase leads to suppression of the IK_{Ca} component of the endothelium-dependent hyperpolarisation to ACh (Dora *et al.*, 2008). Therefore, we hypothesized that activation of β-adrenoceptors with physiological adrenergic agonists may modulate the endothelium-dependent responses in resistance arteries. To confirm this assumption, we investigated the effect of β-adrenoceptor activation on endothelial-dependent dilatation in small resistance arteries from the rat.

5.2 Methods

5.2.1 Rat mesenteric artery isolation and preparation

See Section 2.1 for methods of artery isolation and preparation.

5.2.2 Pressure myography

Arteries were cannulated and pressurized as described in Section 2.2. This method was used to study both local and conducted dilatation responses.

5.2.3 Measurement of local responses

Local dilatation responses were measured using double cannulated vessels (for detailed description see Section 2.2.2). Vessels were preconstricted to 70 - 80% of the maximal tone by PE; in experiments, where adrenergic agonists were perfused via the lumen (luminally), PE was also used to adjust the tone to the similar level as in the control conditions. Cumulative concentration-response curves for ACh (1 nM – 30 μM) were performed by addition of rising concentrations of ACh directly into the bath (abluminally). To allow predominant action on endothelium, studied substances were infused luminally by means of syringe pumps, as described in Section 2.2.1. Adrenergic agonists were perfused luminally for 12-15 minutes before starting the ACh concentration-response. The K_{ATP} channel opener levcromakalim (10 μM) was added at the end of experiment, when endothelium-dependent dilatation was impaired. All inhibitors were applied both luminally and abluminally.

5.2.4 Measurement of spreading responses

Experiments for conducted dilatation studies were performed as described in Section 2.2.3.

5.2.5 Measurement of endothelial cell $[Ca^{2+}]_i$

Endothelial cell $[Ca^{2+}]_i$ was measured as described in Section 2.4, using a cell-permeable Ca^{2+} dye Oregon Green 488 BAPTA-1 AM by means of Olympus IV70 with Andor IQ 1.8.1 spinning disc microscope with 40x water immersion objective (UApo N340, Olympus, Japan) at 0.1 Hz scan frequency.

5.2.6 Data analysis

Data were analyzed as described previously (see Section 2.5).

5.2.7 Drugs and solutions

See Section 2.6 for preparation details. MOPS buffered solution was used throughout in these experiments.

5.3 Results

5.3.1. Pathways of vasodilatation in response to ACh

Arteries were initially preconstricted with PE to achieve 70 - 80% of the maximal tone, and concentration-dependent dilatation in response to abluminal applications of ACh in concentration range from 1 nM to 10 μM was examined (pEC_{50} = 7.23 ± 0.04, *n* = 6; Figure 5.1A). Denudation of the artery resulted in loss of the dilatation (*n* = 2-5; Figure 5.1B). The dilatation was sensitive to the NOS inhibitor L-NAME (100 μM; pEC_{50} = 6.77 ± 0.08, *n* = 5, *P*<0.05), whilst the COX inhibitor indomethacin (10 μM) had no effect (pEC_{50} = 6.74 ± 0.09, *n* = 6, *P*>0.05). The inhibitor of SK_{Ca} channels apamin (50 nM) further attenuated the response (pEC_{50} = 6.23 ± 0.07, *n* = 6, *P*<0.05) and addition of the IK_{Ca} channel inhibitor TRAM-34 (1 μM) completely blocked the remaining vasodilatation (*n* = 5, *P*<0.05).

5.3.2 Effect of luminal application of physiological adrenergic agonists on the vasodilatation evoked by ACh

To examine whether activation of the adrenergic pathways affects endothelium-dependent dilatation, the response to ACh (1nM - 10μM) was explored in the presence of physiological adrenergic agonists. Whilst abluminal application of adrenaline at a concentration that produced a similar level of tone as the control PE-evoked tone (~0.5 μM of adrenaline) exerted statistically insignificant effect on the ACh-evoked dilatation (from pEC_{50} = 7.1 ± 0.02 to pEC_{50} = 6.7 ± 0.03, *n* = 5, *P*>0.05; Figure 5.2A), adrenaline (0.5 μM) and NA (1 μM) perfused luminally significantly shifted the ACh CRC to the right (from pEC_{50} = 7.1 ± 0.02 to pEC_{50} = 6.6 ± 0.02, *n* = 6, *P*<0.05; from pEC_{50} = 7.04 ± 0.03 to pEC_{50} = 6.42 ± 0.3, *n* = 6, *P*<0.05, respectively; Figure 5.2B,C).

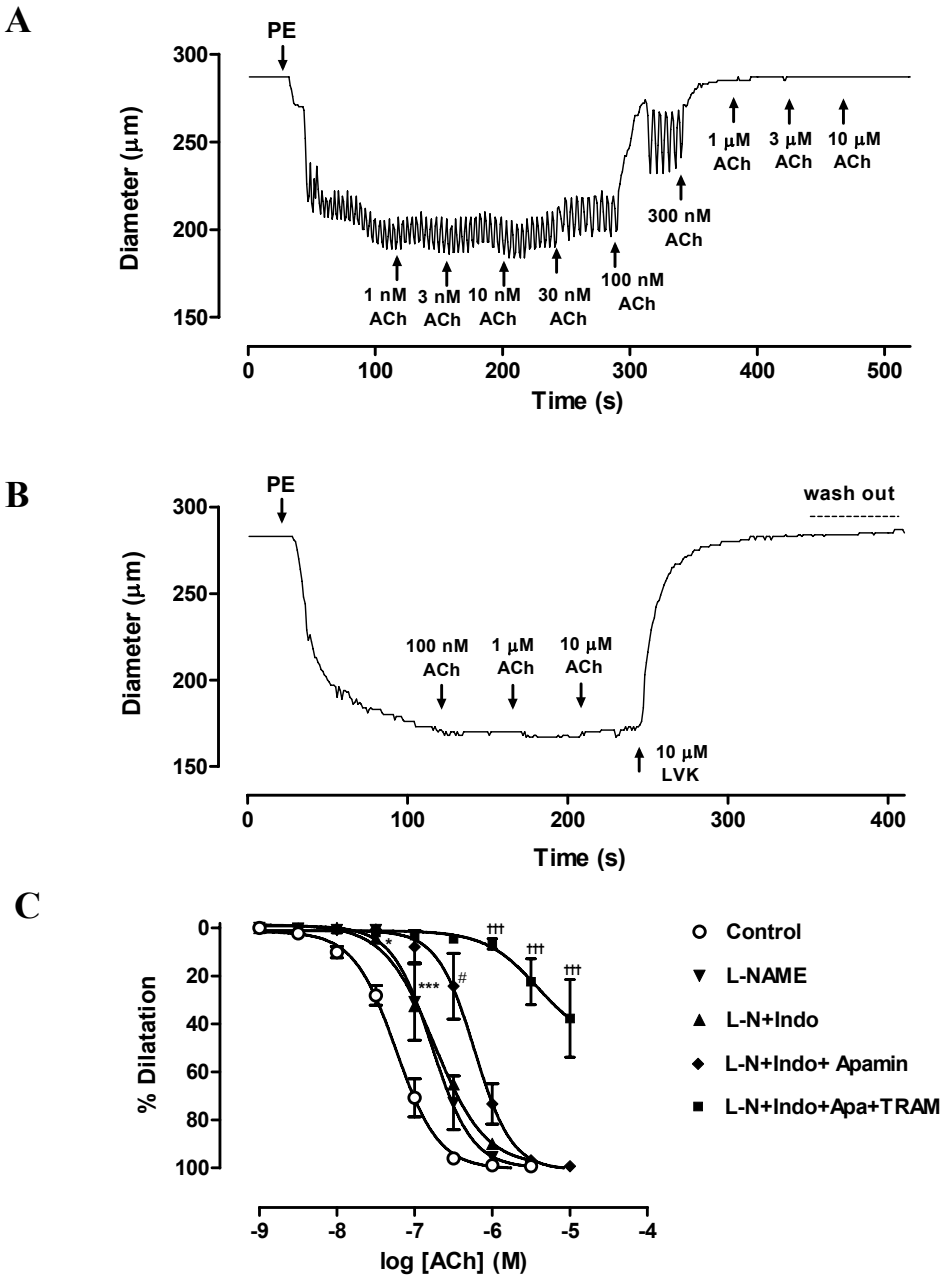


Figure 5.1 Effects of endothelium denudation, eNOS and K_{Ca} channels inhibition on the dilatation to ACh of pressurized small mesenteric arteries

A. Representative trace illustrating responses to cumulative increases of ACh concentrations (from 1 nM to 10 µM) after submaximal precontraction with α_1-adrenoceptor agonist PE (0.5 - 2 µM).

B. Representative trace illustrating the effect of the endothelium denudation on the dilatation to ACh. K_{ATP} channel opener levcromakalim (10 µM) was added at the end of the experiment to evoke endothelium-independent relaxation.

C. Mean (± S.E.M) CRCs representing dilatation to ACh in the presence of L-NAME (100 µM), indomethacin (5 µM), apamin (50 nM) and TRAM-34 (1 µM). *p<0.05, ***p<0.001 vs. control; $^{\#}p$<0.05, vs. L-NAME, $^{\dagger\dagger\dagger}p$<0.001 vs. L-NAME+Indo+Apamin.

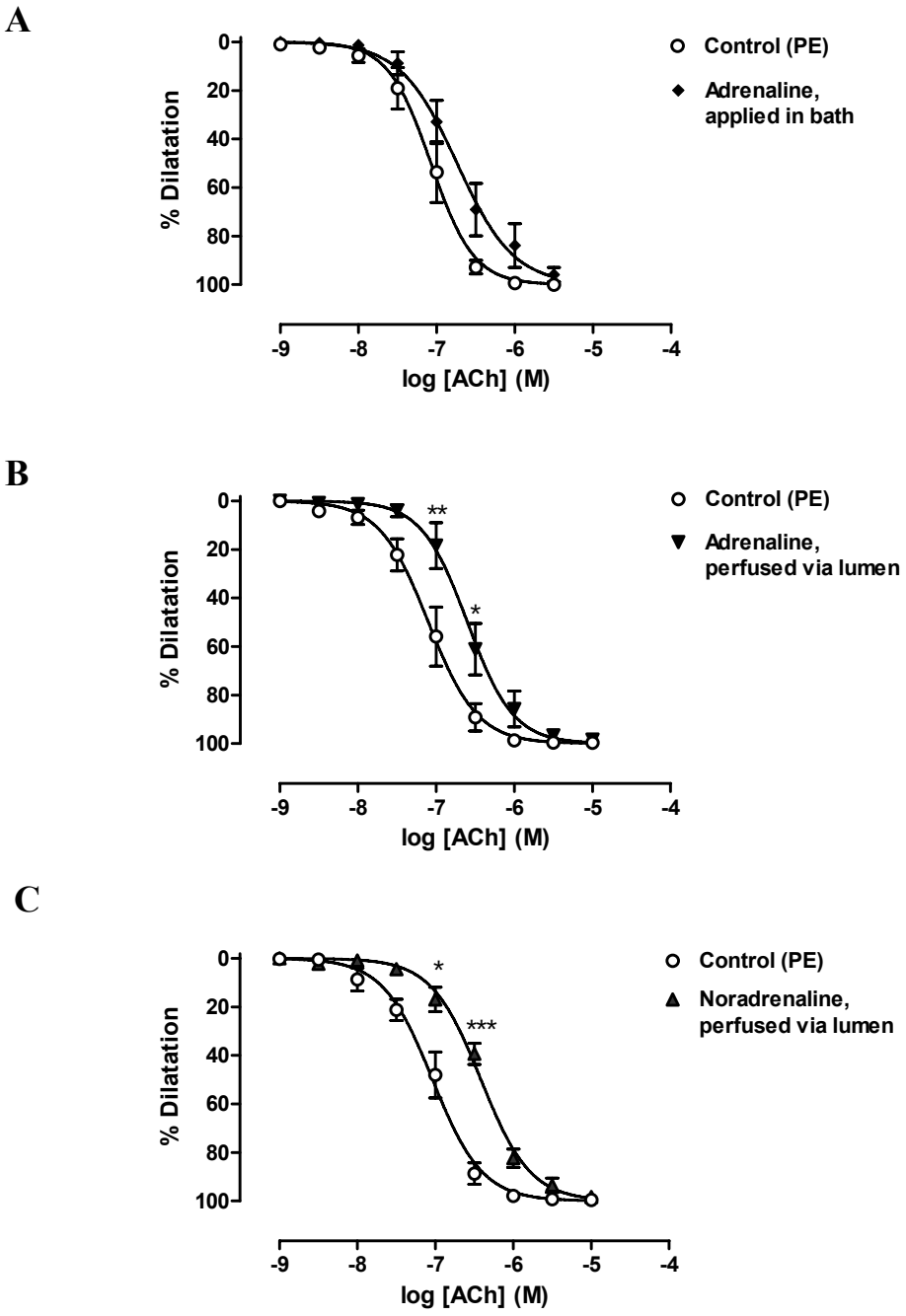


Figure 5.2 Effects of luminal and abluminal application of physiological adrenergic agonists on the dilatation to ACh of pressurized small mesenteric arteries

In paired experiments, precontraction of the vessel with adrenaline had a small, but insignificant effect on ACh CRC (1nM - 10μM; $n = 5$, $p>0.05$; **A**), whilst luminal perfusion of adrenaline (0.5 μM, $n = 5$, $p<0.05$; **B**) or NA (1 μM, $n = 6$, $p<0.05$; **C**) significantly attenuated the dilatation evoked by ACh.
Results shown are the mean ± s.e.mean; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control.

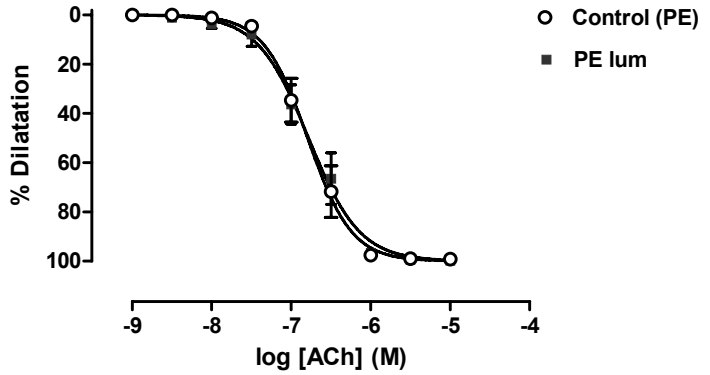
5.3.3 Effect of β-adrenoceptor stimulation on the ACh-mediated vasodilatation

To investigate which adrenoceptor subtype contributes to the inhibition of the dilatation to ACh, selective adrenergic agonists were used. Luminal application of the β-adrenoceptor agonist isoprenaline (1 μM) reversibly inhibited the dilatation to ACh to a similar extent as the nonselective adrenergic agonists adrenaline and NA (from pEC_{50} = 7.15 ± 0.01 to pEC_{50} = 6.44 ± 0.03, n = 6, P<0.05; Figure 5.4A), whilst neither α_1-adrenoceptor agonist PE (0.5 μM; n = 5, P>0.05; Figure 5.3A) nor α_2-adrenoceptor agonist clonidine (1 μM; n = 5, P>0.05; Figure 5.3B) had a statistically significant effect. Moreover, pre-treatment with propranolol (1 μM) eliminated the inhibition of the ACh response by luminal application of adrenaline (n = 4, P>0.05) or NA (n = 4, P>0.05; Figure 5.5B,C). Finally, activation of the downstream effector of β-adrenoceptor signalling, adenylyl cyclase by luminal perfusion of forskolin (0.5-1 μM), mimicked the inhibitory effect of β-adrenergic agonists on the dilatation to ACh (from pEC_{50} = 7.15 ± 0.02 to pEC_{50} = 6.48 ± 0.03, n = 5, P<0.05; Figure 5.4B).

5.3.4. Effect of luminally applied β-adrenergic agonists on the eNOS signalling

To find out whether suppression of eNOS contributed to the β-adrenoceptor-mediated rightward shift of ACh CRC, the action of NOS inhibition on the remaining dilatation was established. Whilst L-NAME (100 μM) attenuated ACh responses in control (n = 5, P<0.05; Figure 5.1C) as well as when PE (pEC_{50} = 6.34 ± 0.02, n = 5, P<0.05; Figure 5.6A) or clonidine (pEC_{50} = 6.18 ± 0.01, n = 6, P<0.05; not shown) were luminally perfused, it did not alter the ACh CRC following luminal application of adrenaline (n = 6, P>0.05; Figure 5.6B), NA (n = 6, P>0.05; Figure 5.6C), isoprenaline (n = 7; P>0.05; Figure 5.7A) or forskolin (n = 4, P>0.05; Figure 5.7B).

A



B

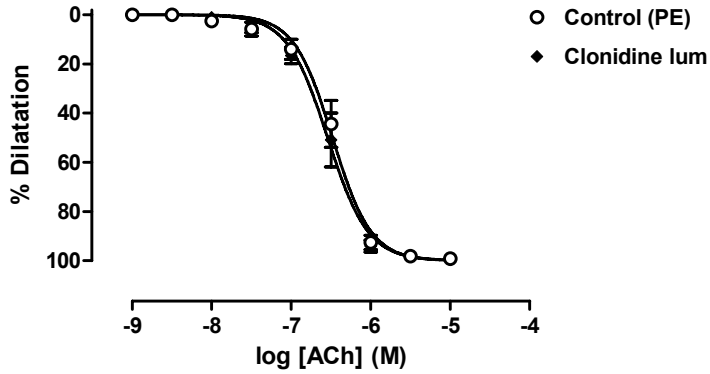
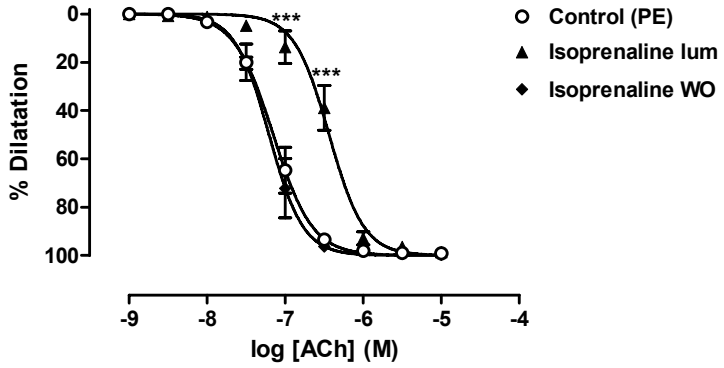


Figure 5.3 Effects of α-adrenergic agonists on the dilatation to ACh in pressurized small mesenteric arteries

In paired experiments, luminal perfusion of selective α_1-adrenergic agonist PE (0.5 μM, $n = 5$, $p<0.05$; **A**) or α_2-adrenergic agonist clonidine ($n = 6$, $p<0.05$; **B**) did not modify the dilatation to cumulative applications of ACh.

Results shown are the mean ± s.e.mean.

A



B

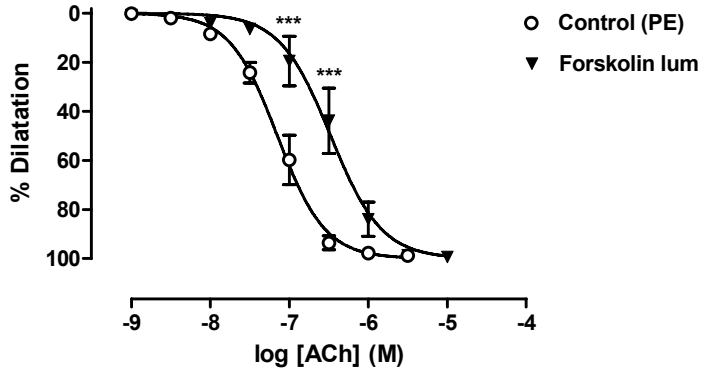


Figure 5.4 Effects of β-adrenergic agonist and forskolin on the dilatation to ACh in pressurized small mesenteric arteries

In paired experiments, luminal perfusion of selective β-adrenergic agonist isoprenaline (1 μM) resulted in inhibition of the dilatation to cumulative applications of ACh (n = 5, p<0.05; **A**). Additionally, the activator of adenylyl cyclase forskolin (1 μM) mimicked the inhibitory effect of isoprenaline on the dilatation (n = 5, p<0.05; **B**).

Results shown are the mean ± s.e.mean; ***p<0.001 vs. control.

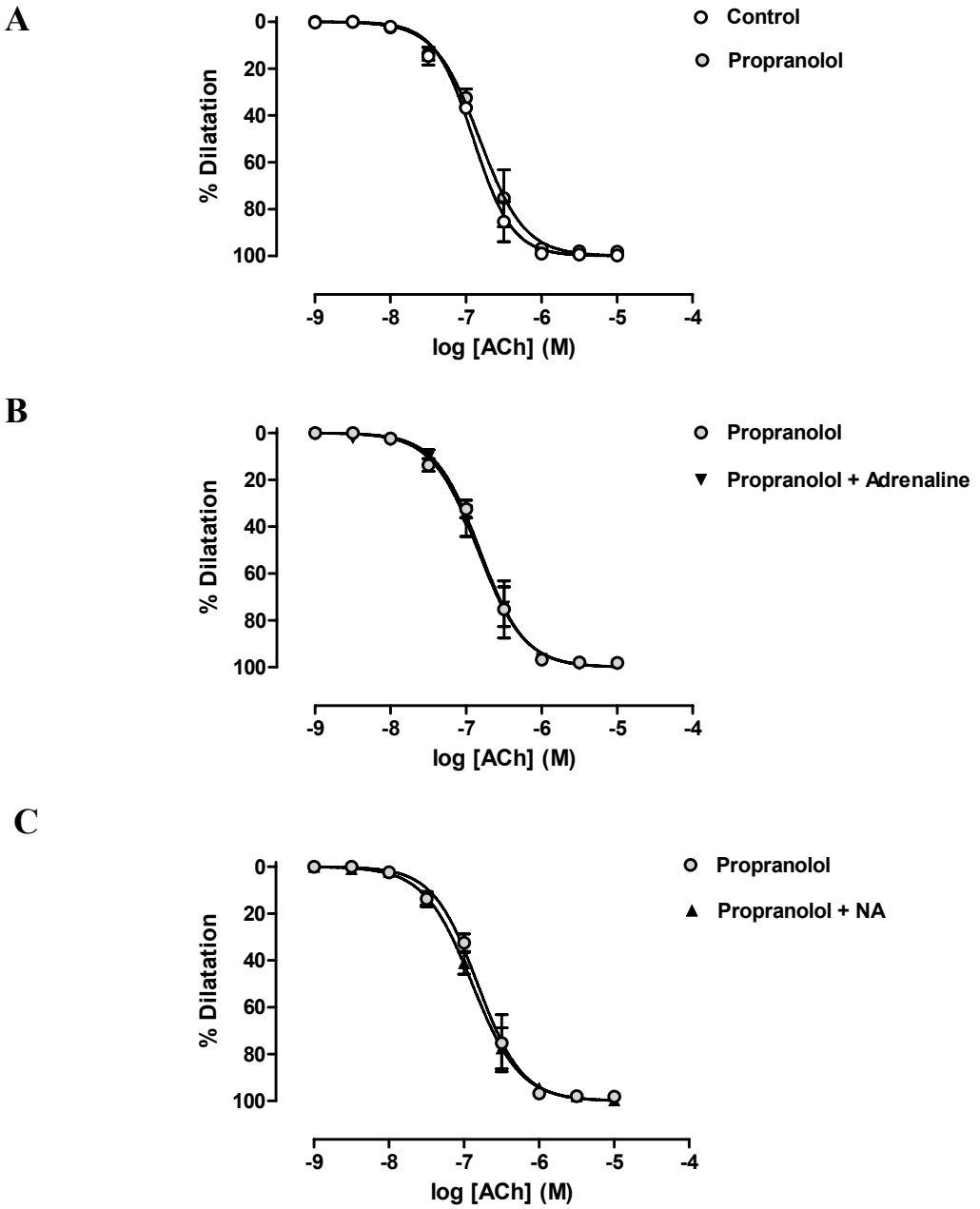


Figure 5.5 Effects of propranolol against the adrenergic agonist-mediated inhibition of the dilatation to ACh in rat pressurized small mesenteric arteries

β-adrenoceptor agonist propranolol (1 μM) did not modify the dilatation to ACh (n = 4; **A**), however, it prevented the inhibition of ACh-induced dilatation by both adrenaline (n = 4; **B**) and NA (n = 4; **C**).

Results shown are the mean ± s.e.mean.

On the other hand, when adrenaline and NA were applied together with propranolol, inhibition of NOS with L-NAME has been revealed ($n = 4$; Figure 5.9 A,B).

As can be seen from Figure 5.1C, dilatation of PE-preconstricted arteries to ACh could be blocked only when both eNOS and K_{Ca} channels were inhibited. Therefore, in the next set of experiments the EDHF pathway was blocked, so the remaining dilatation was enabled only by eNOS function (Andrews *et al.*, 2009). Inhibition of IK_{Ca} channels with TRAM-34 (1 μM) and SK_{Ca} channels with apamin (50 nM), resulted in significant attenuation of the dilatation to ACh ($n = 3$, $P<0.05$). Perfusion of adrenaline (0.5 μM) inhibited the remaining dilatation, and addition of L-NAME had no further effect ($n = 4$, $P>0.05$; Figure 5.7C).

5.3.5. Effect of β-adrenergic signalling on the EDH-mediated vasodilatation

EDH in rat mesenteric arteries is enabled by opening of IK_{Ca} and SK_{Ca} channels. When the EDH pathway is revealed by inhibition of NOS, perfusion of NA shifted the ACh CRC further to the right, showing suppression of K_{Ca} channels function (from $pEC_{50} = 6.83 \pm 0.02$ to $pEC_{50} = 6.2 \pm 0.03$, $n = 4$, $P<0.05$; Figure 5.8A). Application TRAM-34 did not have an additional effect ($pEC_{50} = 6.17 \pm 0.04$, $n = 4$, $P>0.05$). On the other hand, following inhibition of SK_{Ca} channels and eNOS with apamin and L-NAME, respectively, NA, adrenaline or forskolin blocked the remaining dilatation to ACh ($P<0.05$; Figure 5.8B). Application TRAM-34 had an additional effect only at a high concentration of ACh (30 μM; $n = 8$). Importantly, in the presence of propranolol, the dilatation remaining after inhibition of NOS and SK_{Ca} channels became insensitive to adrenergic agonists, but was blocked by TRAM-34 ($n = 4$; Figure 5.9C). This result suggests that the IK_{Ca}, but not SK_{Ca}, channel is predominantly affected following stimulation of β-adrenoceptors.

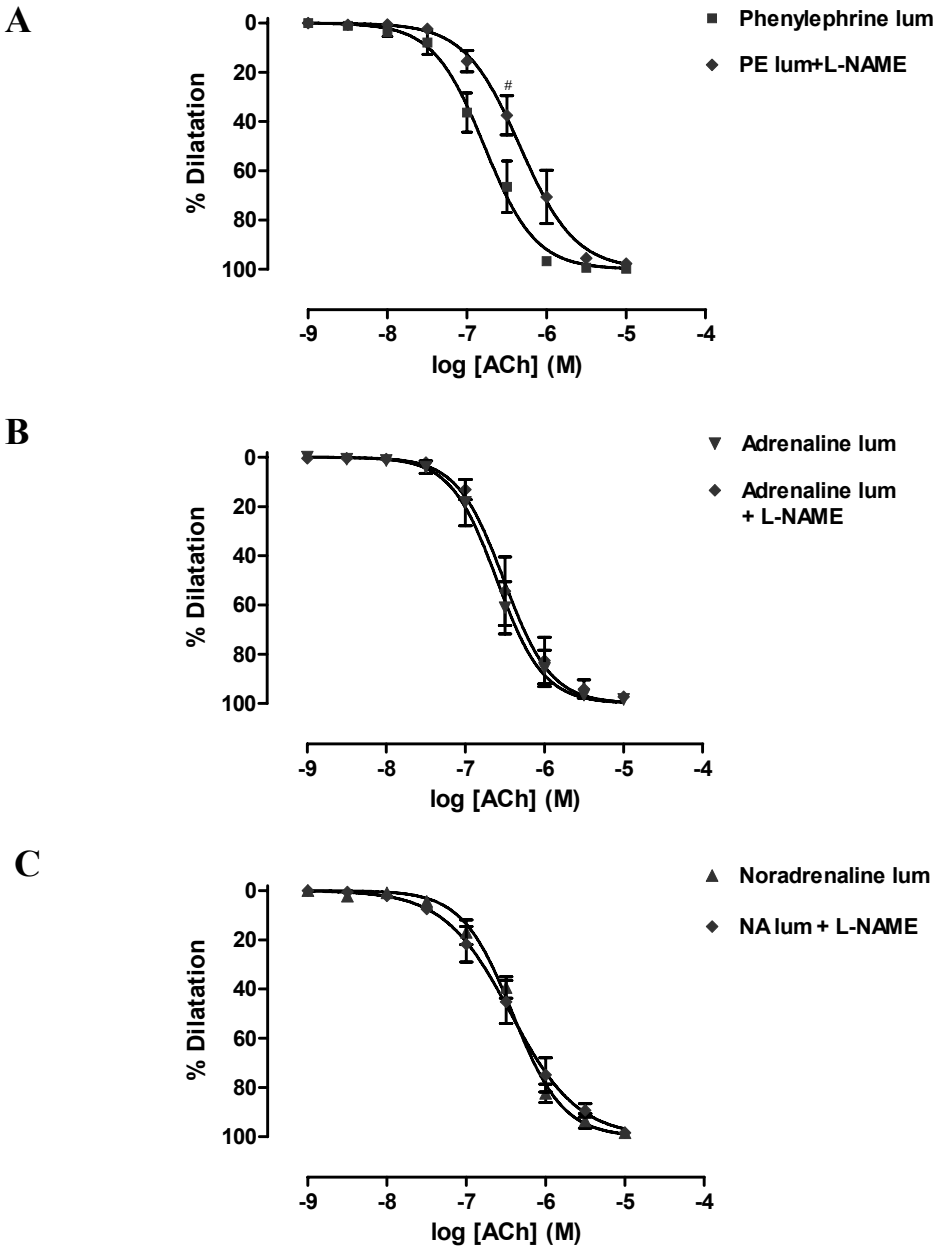


Figure 5.6 Effects of adrenergic agonists on the L-NAME-sensitive component of the dilatation to ACh

In paired experiments, application of L-NAME after luminal perfusion of selective α-adrenergic agonist PE (0.5 μM) resulted in inhibition of ACh-induced dilatation (n = 5, p<0.05; **A**); however, if applied after perfusion of nonselective adrenergic agonists adrenaline (0.5 μM, n = 6, p>0.05; **B**) or noradrenaline (NA; 0.5 μM, n = 6, p>0.05; **C**), L-NAME was ineffective.

Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01, ***p<0.001 vs. control.

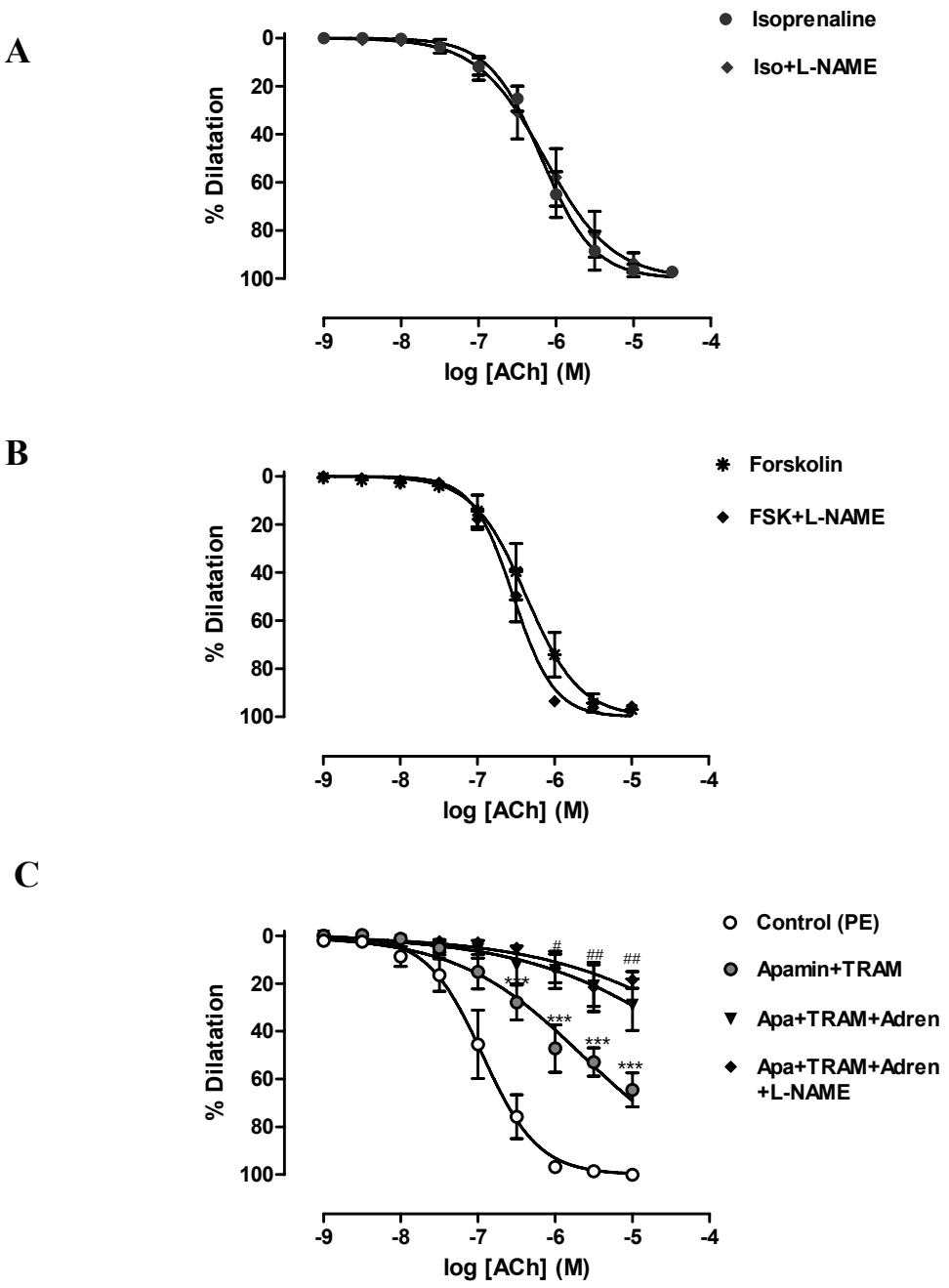


Figure 5.7 Effects of adrenergic agonists and forskolin on the L-NAME-sensitive component of the dilatation to ACh

NOS inhibitor L-NAME (100 μM) was ineffective after luminal perfusion of selective β-adrenergic agonist isoprenaline (1 μM, $n = 7$, $p>0.05$; **A**) or adenylyl cyclase activator forskolin (0.5-1 μM, $n = 4$-8, $p>0.05$; **B**). Application of adrenaline (0.5 μM), after the inhibition of EDH pathway with apamin (50 nM) and TRAM-34 (1 μM), abolished the remaining dilatation to ACh ($n = 3,4$, $p<0.05$; **C**).

Results shown are the mean ± s.e.mean; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. apamin+TRAM-34.

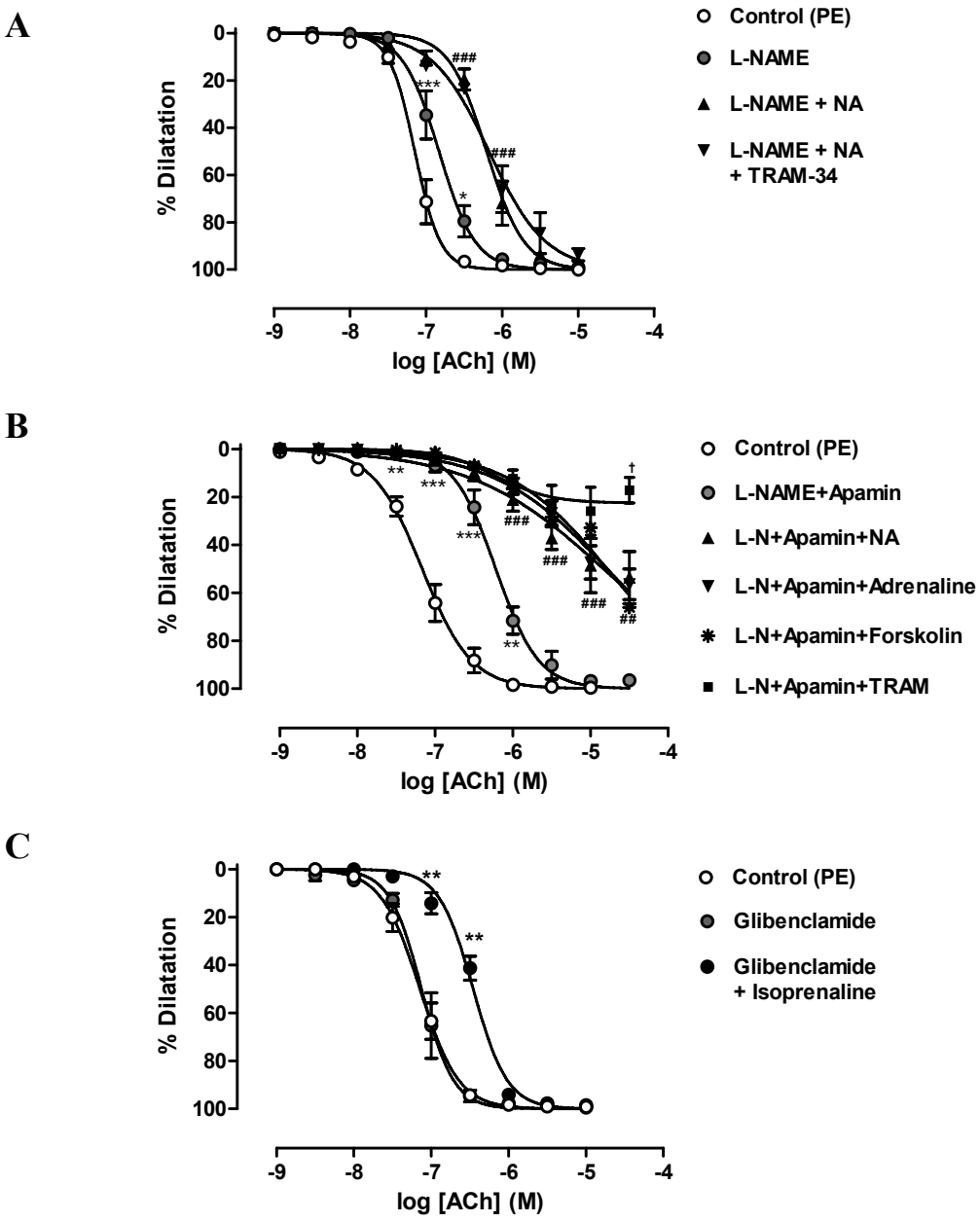


Figure 5.8 Participation of K^+ channels in the β-adrenoceptor mediated inhibition of the dilatation to ACh

A. Effect of luminal perfusion of NA (1 μM) following NOS suppression with L-NAME (100 μM) on dilatation to ACh. IK_{Ca} channel inhibitor TRAM-34 (1 μM) did not affect the remaining dilatation (n = 4, >0.05). **B**. Suppression of TRAM-34 -sensitive component of the dilatation by noradrenaline (NA; 1 μM), adrenaline (0.5 μM) and forskolin (1 μM). **C**. K_{ATP} channel inhibitor glibenclamide (10 μM) failed to affect suppression of the dilatation to ACh induced by isoprenaline (1 μM).
Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01, ***p<0.001 vs. control, $^{\#\#\#}p$<0.001 vs. treated (grey circles).

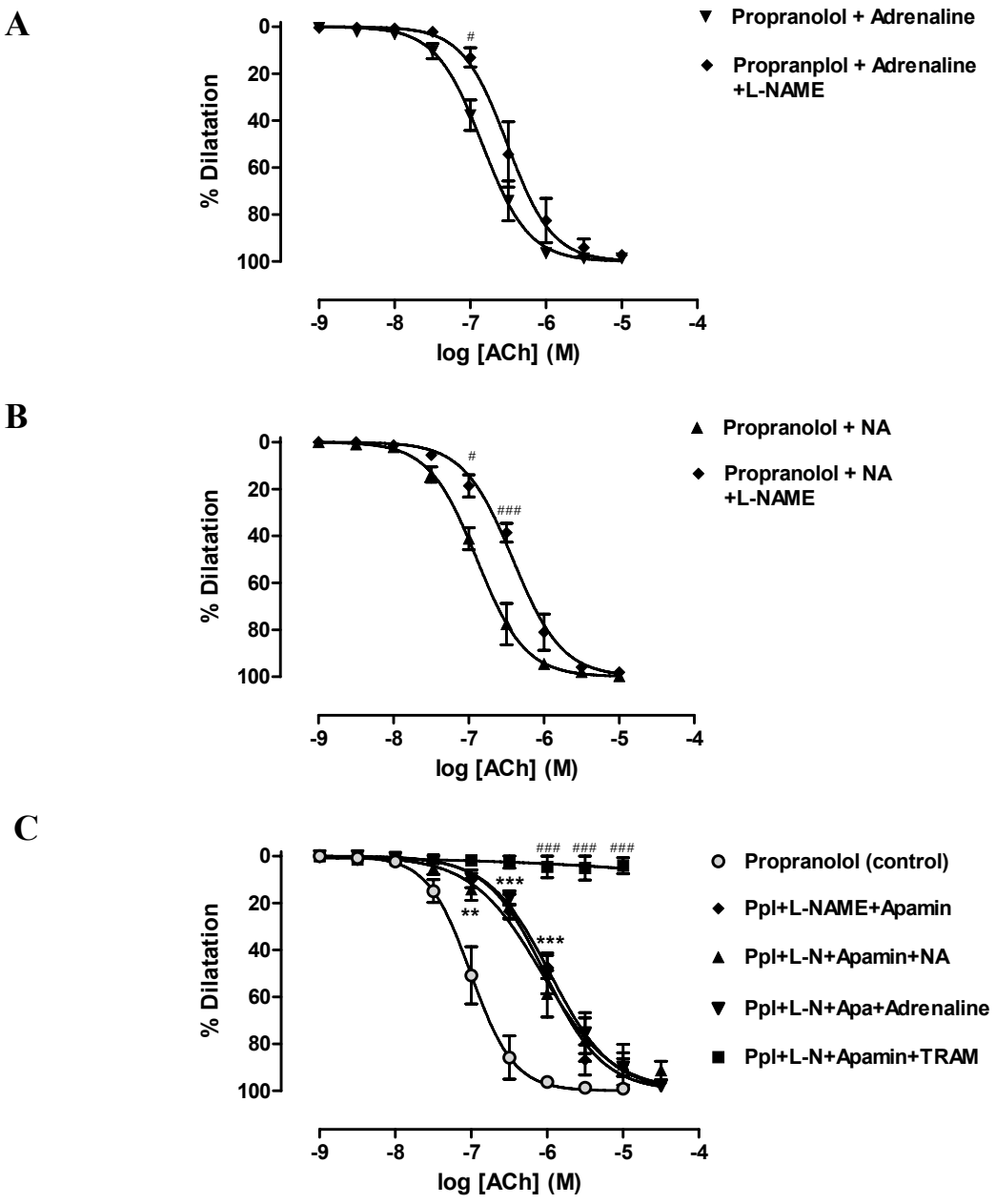


Figure 5.9 Effects of propranolol against the β-adrenoceptor mediated inhibition of eNOS and K_{Ca} signalling

In the presence of β-adrenoceptor antagonist propranolol (1 μM) applied together with the nonselective adrenergic agonists, NOS inhibitor L-NAME (100 μM) lead to a rightward shift of the ACh CRC in the presence of both, adrenaline (**A**) and NA (**B).** Additionally, propranolol prevented the action adrenaline and NA on the TRAM-34 (1 μM)-sensitive component of the dilatation ($n = 4$; **C**).

Results shown are the mean ± s.e.mean; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control (propranolol), $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. luminally perfused adrenergic agonists.

5.3.6. Role of K_{ATP} channel signalling in the β-adrenoceptor-mediated inhibition of the dilatation to ACh

β-adrenoceptor signalling involves hyperpolarization of smooth muscle cells through opening of K_{ATP} channels (Fujii *et al.*, 1999; Garland *et al.*, 2011; Nakashima *et al.*, 1995). To determine whether the resulting smooth muscle hyperpolarization suppressed the EDH-mediated relaxation, we have assessed the effect of the K_{ATP} channel blocker glibenclamide on the β-adrenoceptor-mediated inhibition of the dilatation to ACh. Glibenclamide (10 μM) alone did not affect the dilatation to ACh, moreover, it failed to prevent the inhibition by luminal perfusion of isoprenaline (1 μM; from pEC_{50} = 7.13 ± 0.02 to pEC_{50} = 6.45 ± 0.03, n = 4, P<0.05; Figure 5.8C).

5.3.7. The role of COX in the β-adrenergic inhibition of ACh-mediated vasodilatation

Since β-adrenergic signalling may involve activation of COX (see Chapter 6), an effect of COX inhibitor indomethacin (10 μM) on the β-adrenergic inhibition of ACh response was established. Indomethacin by itself had no effect on the dilatation (from pEC_{50} = 7.13 ± 0.01 to pEC_{50} = 7.15 ± 0.01, n = 5-6, P>0.05; Figure 5.10A), and did not prevent the attenuation of the dilatation by luminal perfusion of isoprenaline (pEC_{50} = 6.7 ± 0.02, n = 5, P<0.05). However, in the presence of indomethacin and isoprenaline, NOS inhibition with L-NAME caused further inhibition of the remaining dilatation (pEC_{50} = 6.38 ± 0.02, n = 5, P<0.05). On the other hand, when eNOS and SK_{Ca} channels were inhibited, indomethacin failed to prevent a significant rightward shift of the ACh CRC mediated by forskolin and isoprenaline (pEC_{50} = 5.95 ± 0.03 and pEC_{50} = 5.9 ± 0.1, respectively, n = 5, P<0.05; Figure 5.10B).

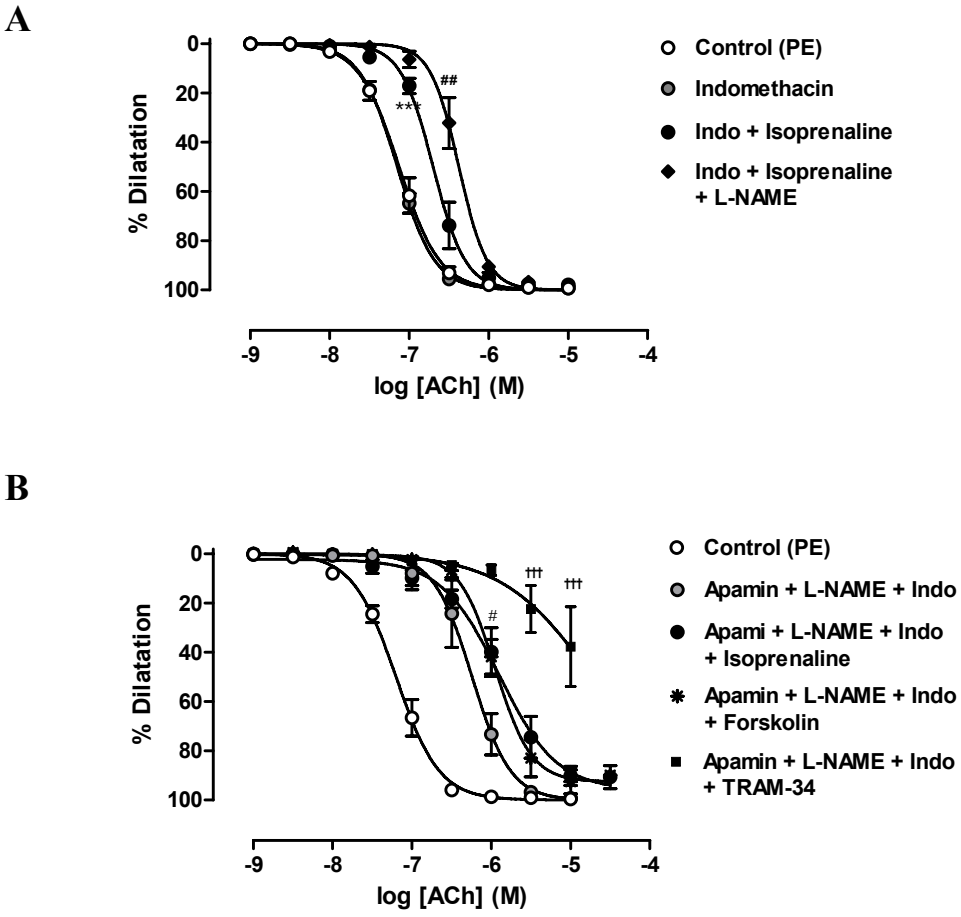
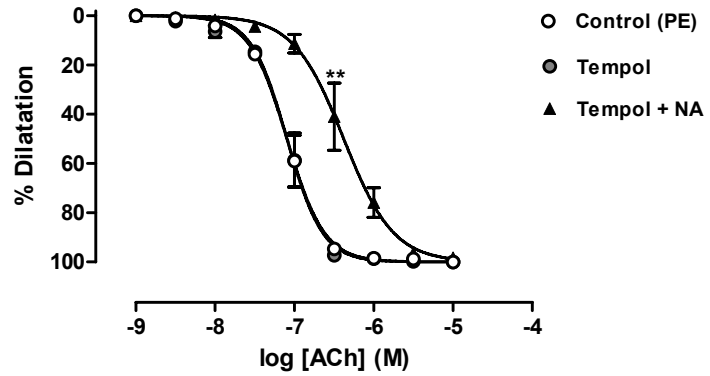


Figure 5.10 Participation of COX in the β-adrenergic-mediated inhibition of the ACh-mediated vasodilatation

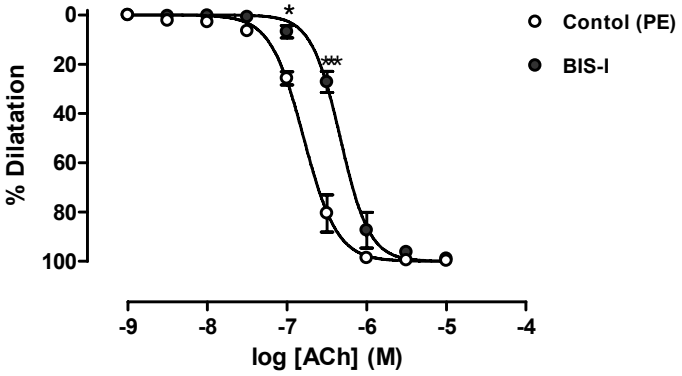
A. Effect of NOS inhibitor L-NAME (100 μM) on the dilatation to ACh, remained after luminally applied β-adrenoceptor agonist isoprenaline (1 μM) in the presence of COX inhibitor indomethacin (10 μM).
B. Effects of luminally applied isoprenaline or forskolin (0.5 μM) on the TRAM-34 (1 μM)-sensitive component of the dilatation in the presence of indomethacin.

Results shown are the mean ± s.e.mean; ***p<0.001 vs. control, $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. treated (grey circles); $^{\dagger\dagger\dagger}p$<0.001 vs. apamin + L-NAME + indomethacin + isoprenaline.

A



B



C

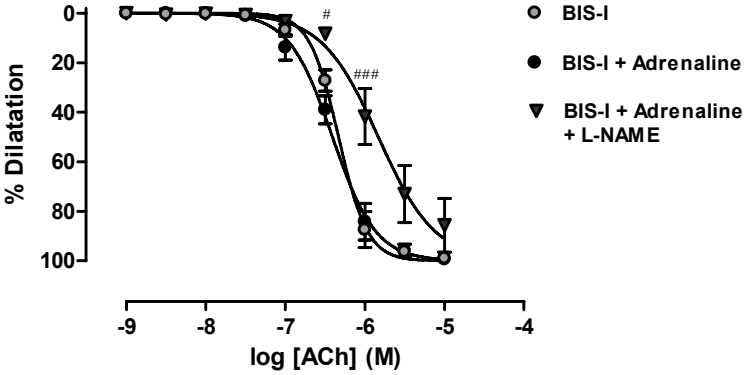


Figure 5.11 A role for ROS and PKC in the β-adrenergic inhibition of the ACh-mediated vasodilatation

A. Effect of ROS scavenger tempol (100 μM) on the isoprenaline (1 μM)-mediated inhibition of ACh dilatation ($n = 3$).
B. Effect of the PKC inhibitor BIS-I (1 μM) on the dilatation to ACh ($n = 8$, $p<0.05$).
C. Effect of the NOS inhibitor L-NAME (100 μM) on the dilatation remaining after luminally applied adrenaline in the presence of BIS-1.
Results shown are the mean ± s.e.mean; $*p<0.05$, $**p<0.01$$***p<0.001$ vs. control, $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. treated (grey circles).

5.3.8. Participation of PKC and ROS in the β-adrenergic inhibition of ACh-mediated vasodilatation

COX signalling is known to involve formation of reactive oxygen species (ROS), which can reduce NO availability. To test the hypothesis that ROS are responsible for the inhibition of the dilatation mediated by β-adrenoceptors, the ROS scavenger tempol (100 μM) was used. However, tempol failed to alter the ACh CRC both in control conditions (from pEC_{50} = 7.09 ± 0.01 to pEC_{50} = 7.09 ± 0.2, n = 3, P>0.05) and after luminal perfusion of NA (1 μM; pEC_{50} = 6.38 ± 0.1, n = 3, P<0.05; Figure 5.11A).

It was reported that products of COX can stimulate the TP receptor (Feletou *et al.*, 2009). One of its downstream signalling enzymes is PKC, which can phosphorylate an inhibitory site on eNOS (Matsubara *et al.*, 2003). PKC inhibitor BIS-I (1 μM) exerted a slight inhibitory action upon dilatation to ACh (from pEC_{50} = 6.79 ± 0.02 to pEC_{50} = 6.33 ± 0.2, n = 8, P<0.05; Figure 5.11B). When applied together with adrenaline, it prevented the rightward shift of the ACh CRC (pEC_{50} = 6.41 ± 0.2, n = 7, P>0.05), moreover, application of L-NAME further attenuated the ACh response (pEC_{50} = 5.83 ± 0.3, n = 7, P<0.05; Figure 5.11C).

5.3.9. Effect of isoprenaline on EC $[Ca^{2+}]_i$

In vascular tissue activation of PKA via the β-adrenoceptor can result in inhibition of store-operated channels (Liu *et al.*, 2005) that may cause reduction of $[Ca^{2+}]_i$ rise in response to ACh, and therefore affect the magnitude of dilatation. Measurement of changes in endothelial cell $[Ca^{2+}]_i$ in paired experiments revealed an insignificant suppression in the response to ACh (0.1-1 μM) in the presence of isoprenaline (1 μM) in comparison to the control (n = 3-7, P>0.05; Figure 5.12).

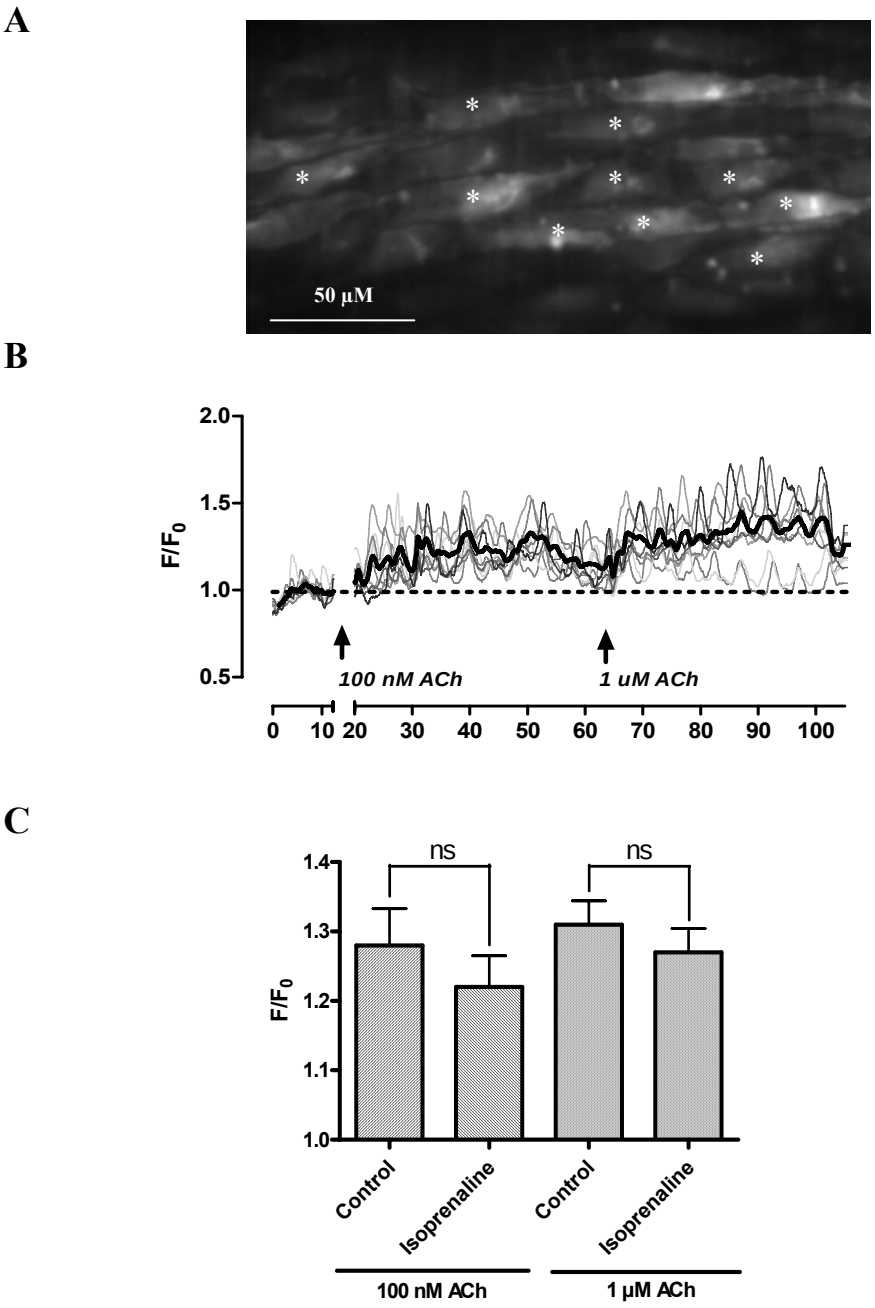


Figure 5.12 Effect of isoprenaline on the ACh-mediated rise in EC $[Ca^{2+}]_i$

A. Representative confocal fluorescence image of endothelial cells loaded with Oregon Green 488 BAPTA-1 AM.
B. Time course of fluorescence intensity changes (F/F_0); individual cells are shown with grey lines (selected cells are labelled with asterisks in A), average intensity is represented by the thick black line.
C. Summarized data of the endothelial cells $[Ca^{2+}]_i$ changes in response to ACh (0.1 - 1 µM). After 10 minutes of incubation with isoprenaline (1 µM) there was an insignificant reduction of the response (n = 3-7).
Result shown are the mean ± s.e.mean; ns p>0.05 vs. control.

5.3.10. Effect of adrenergic stimulation on the spreading dilatation to ACh

Focal application of ACh (1 μM) into the side branch of the triple-cannulated artery caused rapid and extended spread of the dilatation that propagated upstream along the feed artery against the direction of luminal and abluminal flow. Luminal pre-application of 0.5 μM adrenaline led to inhibition of the local response to ACh, in a similar fashion as was observed in the double-cannulated vessels; however, the spread of the dilatation was significantly augmented by adrenaline (n = 8-12; p<0.05; Figure 5.13A). Abluminal application of NA (0.5-1 μM) resulted in a slight improvement of the spreading dilatation, which, however, did not reach significance (n = 4; p>0.05; Figure 5.13A).

To examine the hypothesis that effects of β-adrenergic stimulation on the IK_{Ca}- and eNOS-dependent components of the dilatation to ACh are responsible for the enhancement of the conducted dilatation, we inhibited NOS and IK_{Ca} channels with L-NAME (100 μM) and TRAM-34 (1 μM). Whilst L-NAME did not modify the conducted dilatation (n = 3; p>0.05; Figure 5.13B), TRAM-34 induced a notable augmentation of the propagated dilatation within a short proximity from the site of application (0.5-1.5 mm), which, however, was not significant and faded with distance (n = 3; p>0.05; Figure 5.13C).

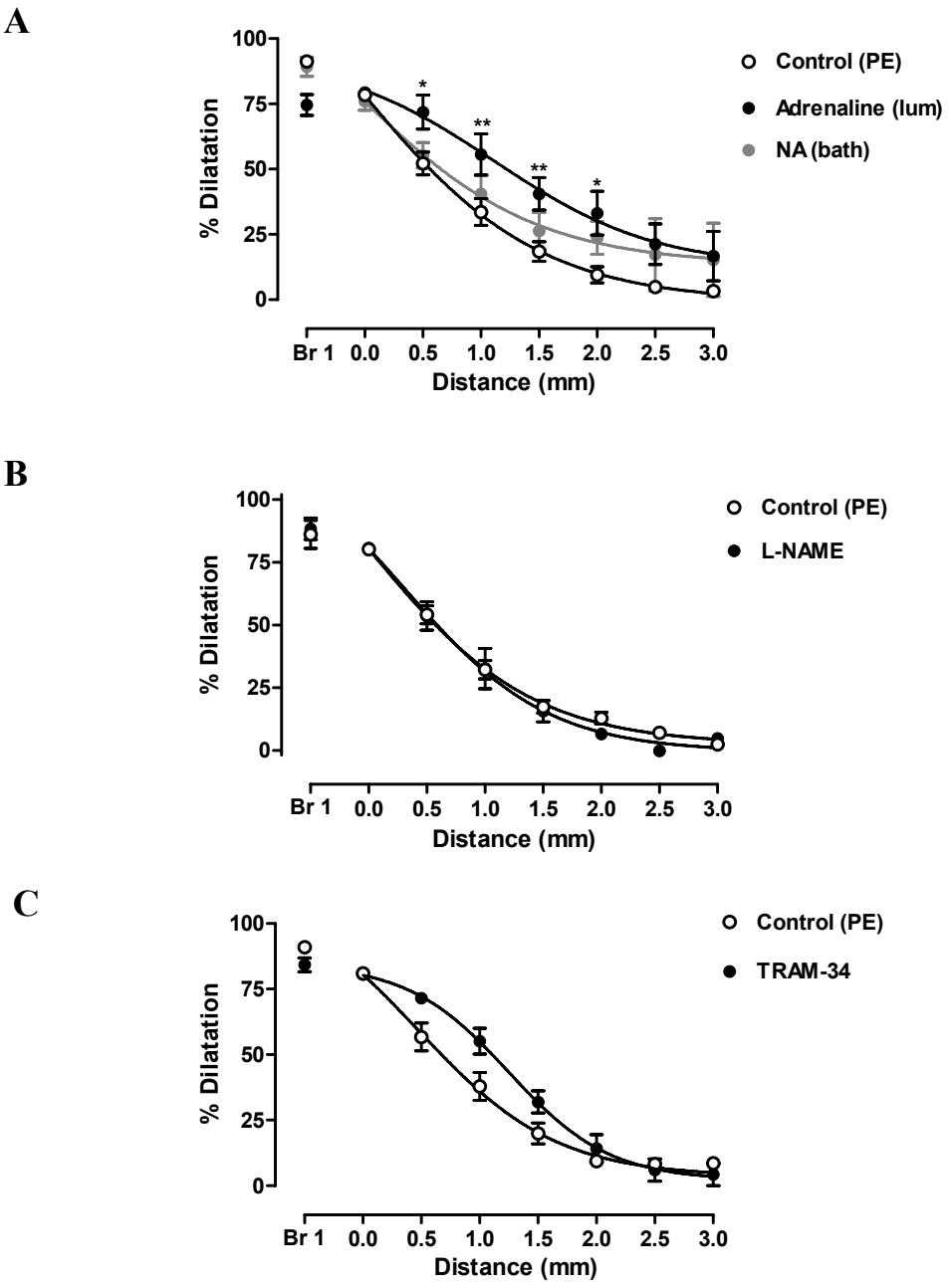


Figure 5.13 Spreading dilatation responses to the luminal application of ACh in the triple-cannulated pressurized small mesenteric arteries

A. Effect of luminal pre-treatment with adrenaline (0.5 μM, $n = 8$) or abluminal application of noradrenaline (NA; 0.5-1 μM, $n = 4$) on the spreading dilatation to ACh (1 μM).
B. Effect of NOS inhibition with or L-NAME (100 μM; $n = 3$) on the spreading dilatation response to ACh.
C. Effect of IK_{Ca} channel inhibitor TRAM-34 (1 μM; $n = 3$) on the spreading response.
Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01 vs. control.

5.4. Discussion

The main findings of this study are that stimulation of the β-adrenoceptors in isolated pressurized small mesenteric arteries of the rat reduces the local dilatation to the muscarinic receptor agonist ACh, whilst improves conducted dilatation. The impaired local dilatation seems to reflect the loss of eNOS and IK_{Ca} components.

The action of adrenaline as a hypertensive drug is very well known (Floras, 1992). Physiologically, adrenaline and NA levels are raised in certain diseases, such as hypertension (Berg *et al.*, 2010; Dietz *et al.*, 1982; Grassi *et al.*, 2010; Klemola *et al.*, 1999; Kuklinska *et al.*, 2010), chronic heart failure (Kubo *et al.*, 1991), primary nephrotic syndrome (Xu *et al.*, 2010) or pheochromocytoma (Galetta *et al.*, 2010; Higashi *et al.*, 2002), leading to impaired vascular function. The sympathoadrenal system is also activated in response to stress (Floras, 1992; Hashiguchi *et al.*, 1997), which is linked to a greater cardiovascular risk status (Chida *et al.*). This creates a basis of the association between "stress" and "hypertension"; however, the precise mechanisms linking raised sympathetic reactivity and the worsening of cardiovascular risk status were not yet determined (Lambert *et al.*, 2010).

One of the explanations arises from the fact that adrenaline, secreted from adrenal medulla and acting on prejunctional adrenoceptors, facilitates release of NA from the sympathetic nerve endings. Additionally, adrenaline can be taken up into the nerve endings and released together with NA, leading to further augmentation of plasma catecholamines concentration and sustained increase in blood pressure (Brown et al., 1981; Floras, 1992).

Although, some studies have demonstrated that physiological or pharmacological increases in plasma adrenaline/NA concentration affects endothelium-dependent responses, in addition to their direct action on smooth muscle α-adrenoceptors. For

example, in patients with increased NA levels endothelium-dependent relaxation was affected (Higashi et al., 2002). Stressful stimuli lead to elevated blood pressure or reduced blood flow and suppression of eNOS function in rats (Bernatowa et al., 2007; Gatenbeck et al., 1987; Yoon et al., 2005). Additionally, arteries from hypertensive human and rats had an impaired eNOS activity or EDH (Higashi et al., 2002; Li et al., 2007; Linder et al., 1990).

Considering this potential physiological relevance, the actions of luminally applied catecholamines on endothelium-dependent relaxation were investigated. We used ACh to activate the endothelial muscarinic M_3 receptors in rat mesenteric arteries (Fujimoto *et al.*, 1991; Rodriguez-Rodriguez *et al.*, 2009; Wu *et al.*, 1997), and found that when applied via the lumen, both NA and adrenaline attenuated ACh–evoked dilatation through a propranolol-sensitive pathway. In contrast, PE and clonidine did not have any effect. Isoprenaline mimicked the inhibitory effect of NA and adrenaline on the ACh-mediated relaxation, confirming the involvement of β-adrenoceptors. These findings correlate with an earlier observation that raised levels of adrenaline may contribute to hypertension via β-adrenoceptors (Borkowski *et al.*, 1985).

To explain this it has been hypothesized that activation of β-adrenoceptors via G_s protein can elevate levels of cAMP and stimulate PKA (Grueb *et al.*, 2008), which, in turn, may modulate eNOS or K_{Ca} channels. Indeed, application of the adenylyl cyclase activator forskolin inhibited the ACh response to a similar extent as isoprenaline introduced luminally. Stimulation of the β-adrenoceptor signalling pathway revealed attenuated endothelium-dependent relaxation by means of diminishing participation of eNOS and IK_{Ca} channels in the relaxation.

There are several possible explanations for the effect of β-adrenergic agonists on IK_{Ca} channels. Firstly, it has been shown that stimulation of AC inhibits IK_{Ca} channels

in rat mesenteric arteries (Dora *et al.*, 2008), guinea-pig duodenum ganglia (Vogalis *et al.*, 2003), and a direct action of PKA on IK_{Ca} was established using Xenopus oocytes (Neylon *et al.*, 2004). Secondly, it may also be possible that activation of the smooth muscle adenylyl cyclase pathway may lead to a requirement for a higher $[Ca^{2+}]_i$ order to reach the same level of tone as in control. A rise in smooth muscle cell $[Ca^{2+}]_i$ in its turn might stimulate Ca^{2+} efflux from the cell, leading to elevation of Ca^{2+} in myo-endothelial space, subsequent stimulation of endothelial CaSR and inhibition of IK_{Ca} (Dora *et al.*, 2008). Finally, we have also investigated the possible role for K_{ATP} channels in the observed inhibition of the vasodilatation. Despite the fact that β-adrenoceptor signalling involves hyperpolarization via K_{ATP} channels, activation of which might have a suppressive effect on the EDH-mediated relaxation, the effect of isoprenaline on the ACh response could not be prevented by K_{ATP} channel blocker glibenclamide.

β-adrenoceptors are generally considered to stimulate eNOS, especially in conduit vessels and cultured endothelial cells (Akimoto *et al.*, 2002; Hashimoto *et al.*, 2006; Huang *et al.*, 1998; Kou *et al.*, 2007; Toyoshima *et al.*, 1998; Zhang *et al.*, 2006b). In perfused mesenteric arteries, stimulation of adrenoceptors with adrenaline was shown to evoke transient NO release and eNOS phosphorylation at Serine 1177, which was sensitive to L-NAME and propranolol (Figueroa *et al.*, 2009b).

On the other hand, in experimental animal models and humans the opposite is true: a chronic increase in adrenergic tone is associated with a reduction in NO availability (Drexler *et al.*, 1994; Gayen *et al.*, 2010; Higashi *et al.*, 2002). It is important to note that in perfused mesenteric arteries NO release in response to β-adrenoceptors stimulation was transient, and lasted for three minutes only (Figueroa *et*

al., 2009b), so it seems possible, that another, perhaps slower, pathway is activated that leads to eNOS inhibition.

In our experiments pre-treatment with β-adrenergic agonists resulted in loss of L-NAME-sensitive component in the dilatation to ACh. This would suggest that β-adrenoceptors cause full activation of eNOS, which could not be further stimulated by muscarinic receptor signalling. However, our previous experiments (see Chapter 4) have shown a lack of effect of L-NAME on the dilatation to β-adrenergic agonists. This may mean that prolonged exposure to β-adrenergic agonists may exert an inhibitory action on eNOS activity. In any case, the NO-mediated component of dilation was prevented.

In the human heart, activation of β_3-adrenoceptors was shown to inhibit eNOS (Napp *et al.*, 2009), but experiments performed in HUVEC failed to demonstrate any changes in eNOS activity after prolonged incubation of the cells with 3 nM of NA (Bachetti *et al.*, 1998). However in HUVECs, β-adrenoceptor stimulation induced a PKA-mediated inhibition of Rho-kinase, which normally suppresses eNOS, thus β-adrenoceptor signalling restored NO production (Seya *et al.*, 2006). In our case, however, PKC inhibition was sufficient to return the L-NAME-sensitive component of the dilatation to ACh, providing evidence for PKC-mediated inhibition of eNOS by adrenaline.

The activity of the endothelial nitric oxide synthase (eNOS) can be upregulated independently of an increase in $[Ca^{2+}]_i$ by the phosphorylation of Ser1177, but results in a low nitric oxide (NO) production (Dimmeler *et al.*, 1999). Recent studies have shown that activation of eNOS is associated with dephosphorylation of Thr495 in the calmodulin (CaM)-binding domain, whilst PKC phosphorylates this inhibitory site at rest (Fleming *et al.*, 2001; Matsubara *et al.*, 2003). Activation of PKC also suppresses arginine transport into cells, resulting in reduced NO production (Krotova *et al.*, 2003;

Venardos *et al.*, 2009). Interestingly, PKC positively modulates neuronal NOS in rat mesenteric arteries (Aras-Lopez *et al.*, 2009). This may explain why stress stimulates NO production in the hippocampus (Zhou *et al.*, 2007).

There is a link between hypertension, PKC-dependent ROS formation and NO uncoupling (Li *et al.*, 2006). This link may explain why in a mouse hypertension model elevated NA/adrenaline concentrations led to ROS formation and NO depletion (Gayen *et al.*, 2010). However, in our experiments the ROS scavenger tempol failed to prevent β-adrenergic–mediated inhibition of dilatation to ACh. There is also an evidence that EDRF release in response to ATP was attenuated after increase of $[cAMP]_i$ due to effect on $[Ca^{2+}]_i$ in cultured endothelial cells (Luckhoff *et al.*, 1990). However, the hypothesis that activation of β-adrenoceptor via PKA can result in inhibition of store-operated channels (Liu *et al.*, 2005), which may reduce $[Ca^{2+}]_i$ rise in response to ACh, appeared not to play a major role in our hands.

There is a lack of evidence of how β-adrenoceptors can activate PKC. Raised cAMP levels were shown to cross talk with PKC through PKA-induced phosphorylation of the enzyme (Wooten *et al.*, 1996). The most recent publication supports this cross-talk by demonstrating the isoprenaline-mediated activation of PKC via the cAMP-binding protein Epac (Duquesnes *et al.*, 2010). But it may be possible, that PKC activation is indirect, and occurs via another pathway.

In bovine aortic endothelial cells, a rise in cAMP up-regulated COX-2 in a PKA-dependent manner (Samokovlisky *et al.*, 1999). COX-2 can be upregulated by PKA in osteoblasts as well (Choudhary *et al.*, 2004). There is also evidence showing that β-adrenoceptors can stimulate COX-1,2 in rat aorta and cremaster arterioles (Kang *et al.*, 2007). On the other hand, exaggerated COX signalling is known to be associated with endothelial dysfunction (Bratz *et al.*, 2004; Feletou *et al.*, 2009).

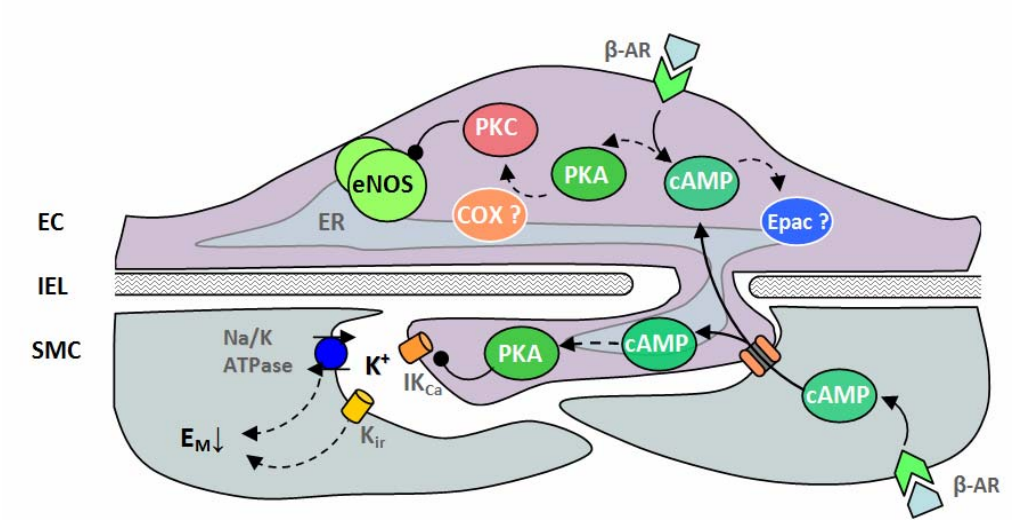


Figure 5.14 Diagram illustrating proposed pathway for β-adrenoceptor-mediated suppression of endothelial cell signalling

Stimulation of β-adrenoceptors located on smooth muscle or endothelial cell promotes cAMP production by adenylyl cyclase. The cAMP can activate both PKA (green) and Epac (blue). PKC (pink), which may be upregulated by PKA, is able to phosphorylate Thr495 site on eNOS leading to suppression of NO production. On the other hand, PKA can phosphorylate thus inhibit IK_{Ca} channels, affecting the EDHF pathway. COX may also play a role in the eNOS inhibition; however, the signalling pathway is not yet determined.

In some studies, after inhibition of NOS with L-NAME, the ACh-dependent response in rat mesenteric arteries was further attenuated by indomethacin (Ben Cheikh *et al.*, 2002). Our experiments, though, do not support this evidence. Indomethacin failed to inhibit ACh-dependent relaxation; moreover, it reduced the suppressive effect of β-adrenoceptor stimulation via revealing a L-NAME-sensitive component. The effect on IK_{Ca} had a tendency of reduction too, however, both, isoprenaline and forskolin still evoked significant suppression of the TRAM-34-sensitive component of the dilatation in the presence of indomethacin.

COX function has been shown to inhibit NOS signaling in rat mesenteric arteries (Bratz *et al.*, 2004; Xavier *et al.*, 2009), but the precise mechanism is still unknown. Both COX subtypes participate in production of contractile prostanoids by the endothelium; all the prostanoids can bind to TP receptor with different affinities (Feletou *et al.*, 2009). TP receptor activation can lead to Rho kinase-dependent inhibition of NO production (Liu *et al.*, 2009; Ming *et al.*, 2002), or to PKC-mediated uncoupling of eNOS (Perez-Vizcaino *et al.*, 1997; Zhang *et al.*, 2010). Inhibition of eNOS in turn triggers release of additional contracting factors from COX, which can again activate TP receptor (Ben Cheikh *et al.*, 2002). This mechanism may explain why activation of the TP receptor can lead to release of prostacyclin from endothelial cells (Hunt *et al.*, 1992), leading to hypertension and atherosclerosis (Feletou *et al.*, 2009). This pathological positive feedback loop may be created in response to β-adrenoceptor signaling in rat small mesenteric arteries.

In addition, a stable thromboxane A_2 mimetic U46619 has been shown to inhibit SK_{Ca} channels in rat mesenteric and cerebral arteries (Crane *et al.*, 2004; McNeish *et al.*, 2007), when used in high concentrations or following repetitive applications, the whole EDH response to ACh was blocked (Crane *et al.*, 2004; Plane *et al.*, 1996). These data support a pathophysiological significance of endothelial TP receptor signalling.

Whilst attenuating the magnitude of the endothelium-dependent relaxation at the site of application, β-adrenergic stimulation had a beneficial effect on the conducted dilatation response. The fact that abluminal application was less effective than luminal perfusion may be explained by a predominant effect on the endothelial cell layer. This goes in accordance with recent evidence that PKA signalling enhances interendothelial gap junctional communication (Griffith *et al.*, 2002; Popp *et al.*, 2002). It has also been shown that a downstream effector of adenylyl cyclase, Epac, induces connexin

phosphorylation and function in rat cardiomyocytes (Duquesnes *et al.*, 2010). On the other hand, the effect of β-adrenergic stimulation can be explained by loss of current dissipation due to inhibition of IK_{Ca}, which was partly confirmed by our observation that TRAM-34 tended to augment propagated vasodilatation within a short proximity from the site of application (0.5 - 1.5 mm). Conducted dilatation may also be enhanced by release of COX products, which can activate TP receptor. As can be seen from Chapter 6, precontraction with the TP agonist U64419 also resulted in enhancement of the spreading response.

Lastly, it has been recently shown that treatment with propranolol or subtype selective β-adrenoceptor blockers helps to attenuate arterial blood pressure and increase NO bioavailability in animals and humans (Buval'tsev *et al.*, 2003; Gupta *et al.*, 2008; Priviero *et al.*, 2007; Reiter, 2004; Wenzel *et al.*, 2009); however, the mechanisms involved were still poorly understood. We can speculate that the effect of β-adrenergic antagonists may at least in part be a result of inhibition of vascular β-adrenoceptors, which prevents the blood plasma catecholamine-mediated rise in endothelial cell cAMP levels, and may therefore attenuate the inhibition of eNOS and IK_{Ca} channels. On the other hand, it is worth remembering that intake of β-adrenoceptor blockers may diminish the beneficial effect of β-adrenoceptor signalling on the conducted vasomotor response.

Chapter 6. β-adrenoceptors and a possible endothelium-derived contracting influence

6.1 Introduction

Endothelial dysfunction is implicated in various cardiovascular diseases, and is particularly important during ageing and development of hypertension. It is generally defined as a decrease in the capacity of the endothelium to dilate blood vessels in response to physical and chemical stimuli and can be associated with the release of endothelium-derived contracting factors (EDCFs), which attenuate the effects of endothelium-derived dilatation. The major factors that contribute to the endothelium-derived contraction are metabolites of arachidonic acid that can activate prostanoid receptors expressed in both endothelial and smooth muscle cell layers (Feletou *et al.*, 2010; Wong *et al.*, 2010b).

Arachidonic acid is a product of phospholipase A_2 (PLA). The Ca^{2+}-dependent form of PLA is activated when $[Ca^{2+}]_i$ rises in response to cell stimulation, whilst Ca^{2+}-independent PLA is regulated by phosphorylation. Notably, the Ca^{2+}-independent form has been found to be mainly involved in the EDCF release by aortic endothelial cells from hypertensive rats (Wong *et al.*, 2010a). Regardless of the source, released arachidonic acid can then be metabolized by cyclooxygenase (COX), which produces endoperoxide (PGH_2) that may undergo further transformations by specific synthases, creating a range of prostanoids. Prostacyclin, prostaglandins D, E_2, $F_{2\alpha}$ and thromboxane A_2, all are able to activate TP receptor with different potencies; however, although thromboxane A_2 is the most potent agonist, prostacyclin seems to be the principal metabolite, which acts on the TP receptor and thereby promotes a rise in blood pressure and the development of endothelial disfunction associated with numerous pathophysoplogical processes in the vasculature of humans and rats (Feletou *et al.*, 2010; Wong *et al.*, 2010b).

On the other hand, β-adrenoceptor-mediated vasodilatation is also known to be altered in age and disease. It was therefore proposed that the age-related reduction in β-adrenoceptor responsiveness may lead to chronic blood pressure elevation and induce hypertension development (Blankesteijn *et al.*, 1996; Borkowski *et al.*, 1992). Indeed, the reduced vasodilatation to isoprenaline was observed in aorta from old rats (Borkowski *et al.*, 1992; Gomez *et al.*, 2008; Kang *et al.*, 2007), as well as in aorta (Borkowski *et al.*, 1992; Gomez *et al.*, 2008), femoral and mesenteric arteries (Fujimoto *et al.*, 2001) of old spontaneously hypertensive rats (SHRs), but it was almost unaltered in mesenteric arteries from young SHRs (Blankesteijn *et al.*, 1996). The decrease in β-adrenoceptor signalling was proposed to be mediated by reduced receptor density and G-protein coupling (Gurdal *et al.*, 1995), attenuated cAMP synthesis or impaired PKA function (Deisher *et al.*, 1989), although it might also result from the endothelial dysfunction.

It is still subject to debate whether the endothelium participates in the dilatation in response to β-adrenergic stimulation. Whilst some research groups support the involvement of endothelial nitric oxide synthase (eNOS) (Figueroa *et al.*, 2009; Graves *et al.*, 1993; Kozlowska *et al.*, 2003), others demonstrate no effect of eNOS inhibition on the dilatation to β-adrenergic agonists (Blankesteijn *et al.*, 1993; Briones *et al.*, 2005a). Intriguingly, removal of the endothelium in our experiments (see Chapter 4) as well as in some previous publications (Iwatani *et al.*, 2008) lead not to inhibition, but to augmentation of the dilatation in rat mesenteric arteries. This could be explained by release of some contractile factors by the endothelium, which may counteract the β-adrenergic-mediated dilatation (Iwatani *et al.*, 2008).

Tissue levels of COX metabolites, such as prostacyclin (Kang *et al.*, 2007; Numaguchi *et al.*, 1999), $PGF_{2\alpha}$ (Briones *et al.*, 2005c), PGF_2 (Matz *et al.*, 2001) and

thromboxane A_2 (Briones *et al.*, 2005; Garcia-Redondo *et al.*, 2009; Kang *et al.*, 2007; Matz *et al.*, 2001) were found to be increased with age or in hypertensive rat models. The expression of the enzyme in endothelial cells was enhanced in arteries from SHRs (Kang *et al.*, 2007; Numaguchi *et al.*, 1999), not altered (Briones *et al.*, 2005b) or augmented (Matz *et al.*, 2000) in normotensive old rats. Recently a role for COX was revealed in the aging-related decrease in responsiveness to β-adrenergic agonists in rat aorta (Kang *et al.*, 2007); however, the mechanism by which the increased COX signalling influences β-adrenoceptor relaxation in small resistance arteries that are predominantly involved in the regulation of peripheral blood pressure, is not clear.

Also, apart from local effects, β-adrenergic signalling can lead to a conducted vasomotor response (Garland *et al.*, 2010b), an important event that may significantly enhance the effects of physiological regulation of the blood flow (Dora, 2010; Garland *et al.*, 2010a). The question of how the conducted vasodilatation can be modulated by TP receptor, to our knowledge, has not yet been addressed.

Therefore, we decided to examine β-adrenergic responsiveness in young (12-14 weeks) Wistar, old (6 months) Wistar and old spontaneously hypertensive rats, and evaluate the role of COX signalling in this dilatation to isoprenaline. We also have elucidated the effect of TP receptor stimulation on both local and conducted vasodilatation in rat small mesenteric arteries.

6.2 Methods

6.2.1 Rat mesenteric artery isolation and preparation

In this study, 12 weeks old Wistar, 6 months old Wistar and 6 month old spontaneously hypertensive rats (in SHRs, mean blood pressure (MAB) = 171.8 ± 2.8, n = 5 in comparison to MAB = 131.8 ± 3.04, n = 3 in the age-matching Wistar rats) were used. For the detailed description of how arteries were isolated and prepared, see Section 2.1.

6.2.2 Pressure myography

Arteries were cannulated and pressurized as described in Section 2.2. Pressure myography was used to study both local and spreading responses. To obtain denuded arteries, pressure was decreased and an air bubble was perfused through the artery using the syringe pump system. The lumen was then washed and 70 mm Hg pressure was re-introduced.

6.2.3 Measurement of local responses

Local dilatation responses were measured using double cannulated vessels (for methods see section 2.2.2). Vessels were preconstricted to 70-80% of maximal tone by PE or U46619, where indicated. α_1-adrenoceptor inhibitor prazosin was used to reveal dilatation evoked by nonselective adrenergic agonists. Cumulative concentration-response curves (CRCs) to agonists were performed by addition of rising concentrations of agonist directly in bath (abluminally) or perfused via lumen by means of syringe pumps, as described in Section 2.2.1.

6.2.4 Measurement of spreading responses

Experiments for conducted dilatation studies were performed as described in Section 2.2.3.

6.2.5 Data analysis

Data were collected and analyzed as described previously (see Section 2.5).

6.2.6 Drugs and solutions

All drugs and solutions were prepared as detailed previously (see Section 2.6). MOPS buffered solution was used throughout in these experiments. Solutions containing 10 μM indomethacin had their pH additionally adjusted to 7.4 ± 0.02.

6.3 Results

6.3.1 Effect of COX inhibition on the dilatation to ACh

ACh (1 nM - 10 μM) concentration-dependently dilatated pressurized small mesenteric arteries preconstricted with PE from 12 week old (young) Wistar rats, 6 month old (old) Wistar rats and old SHRs. Arteries from young and old Wistar rats fully dilatated with a slight, but significantly greater potency compared to arteries old SHRs (pEC_{50} = 7.13 ± 0.01, n = 6; and pEC_{50} = 7.37 ± 0.02, n = 4, subsequently; p<0.01; Figure 6.1A,B). The E_{max} to ACh in arteries from SHRs was only 87.3 ± 3.5% at 1 μM ACh, and higher concentrations evoked a slight constriction to 78.2 ± 7.7% dilatation at 10 μM ACh (pEC_{50} = 7.0 ± 0.12, n = 4; Figure 6.1C). Whilst indomethacin (10 μM) had no effect in both young (pEC_{50} = 7.15 ± 0.01, n = 5) and old Wistar rats (pEC_{50} = 7.37 ± 0.03, n = 4), it enhanced the dilatation to ACh in arteries from old SHRs (E_{max} = 98.0 ± 1.1%, pEC_{50} = 7.18 ± 0.04, n = 4; Figure 6.1A,B,C).

6.3.2 Effects of COX and eNOS inhibition on the dilatation to isoprenaline

The β-adrenoceptor agonist isoprenaline (1 nM- 10 μM) produced different effects on each group of rats. Whilst it almost maximally dilatated the arteries from young Wistar rats (E_{max} = 96.6 ± 2.4%; pEC_{50} = 7.14 ± 0.1, n = 7; Figure 6.2A), the dilatation of arteries from old rats was reduced (E_{max} = 74.4 ± 14.8%; n = 4; Figure 6.2B), and there was no notable dilatation to isoprenaline in the SHRs (E_{max} = 5.9 ± 4.3%, n = 2; Figure 6.2C).

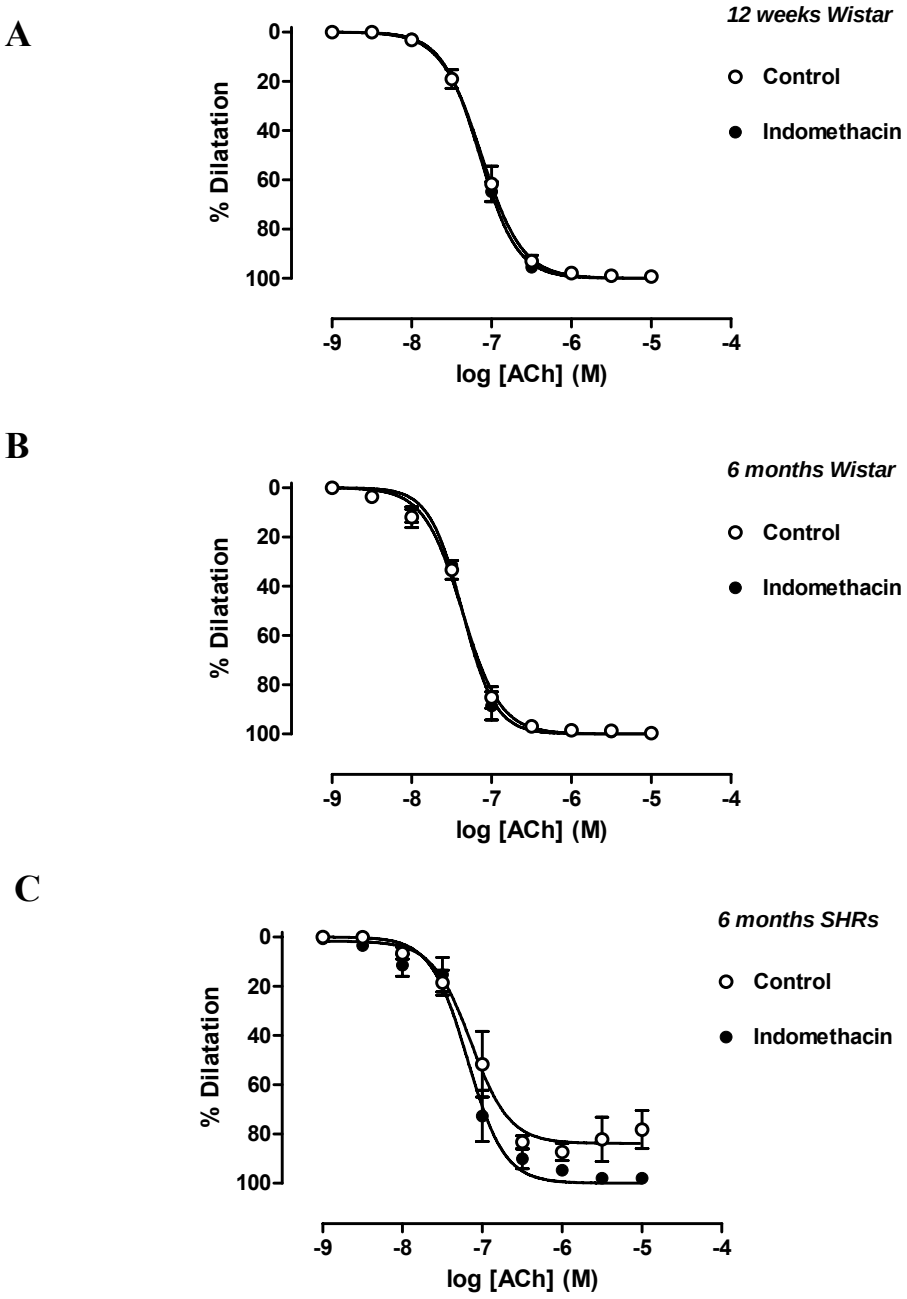


Figure 6.1 Effect of indomethacin on the concentration-dependent dilatation to ACh of rat pressurized small mesenteric arteries

A. In the presence of tone induced by α_1-adrenoceptor agonist PE, arteries of 12 week old Wistar rats dilated to rising concentrations of ACh (1 nM – 1 μM; n = 6). The COX inhibitor indomethacin (10 μM) did not affect the response (n = 5).
B. Dilatation to ACh in arteries from the 6 months old rats was also unaffected by indomethacin (n = 4).
C. High concentrations of ACh (1 μM -10 μM) evoked less dilatation (and even contraction) in arteries from 6 month old SHRs (n = 4).
Results shown are the mean ± s.e.mean.

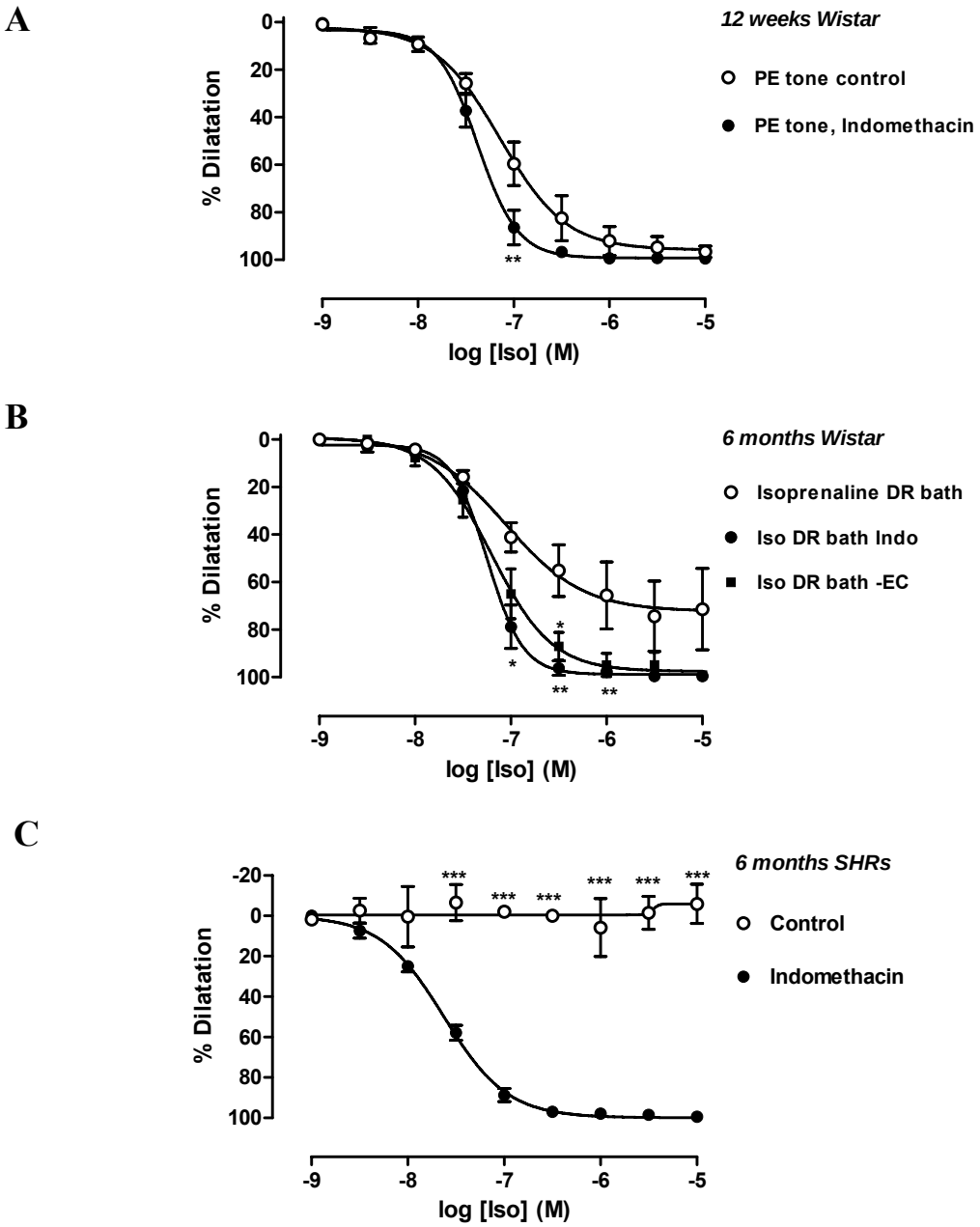


Figure 6.2 Effect of indomethacin on the concentration-dependent dilatation to isoprenaline of rat pressurized small mesenteric arteries

A. Isoprenaline dilated arteries of 12 week old Wistar rats, preconstricted by the α_1-adrenoceptor agonist PE. The COX inhibitor indomethacin (10 μM) slightly enhanced the response ($n = 7$).
B. Reduced dilatation to isoprenaline in arteries from 6 months old Wistar rats was restored by indomethacin or endothelium denudation ($n = 4$).
C. Isoprenaline failed to dilate arteries from 6 months SHRs, and indomethacin unmasked the dilatation ($n = 2$).
Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01, ***p<0.001 vs. control.

Indomethacin had a slight effect on the dilatation to isoprenaline in arteries from young Wistar rats (E_{max} = 99.5 ± 0.3%; pEC_{50} = 7.38 ± 0.04, *n* = 7; Figure 6.2A); however it improved the response in old Wistar rats (E_{max} = 99.6 ± 0.4%; pEC_{50} = 7.25 ± 0.03, *n* = 4; Figure 6.2B) and fully restored the dilatation in the old SHRs (E_{max} = 99.5 ± 0.5%; pEC_{50} = 7.63 ± 0.01, *n* = 2; Figure 6.2C). Endothelium denudation also improved the response to isoprenaline in old Wistar rats (E_{max} = 99.2 ± 0.8%; pEC_{50} = 7.21 ± 0.07, *n* = 4; Figure 6.2B).

Whilst the NOS inhibitor L-NAME attenuated the dilatation to ACh (see Figure 5.1C), it had no effect on the dilatation to isoprenaline (see Figure 4.9C). To elucidate if inhibition of COX can unmask the eNOS-dependent component of the dilatation, we examined the effect of L-NAME (100 μM) applied together with indomethacin. CRCs to isoprenaline remained unmodified after application of L-NAME in all groups, young Wistar rats (pEC_{50} = 7.4 ± 0.04, *n* = 2; *p*>0.05; Figure 6.3A), old Wistar rats (pEC_{50} = 7.17 ± 0.03, *n* = 4; *p*>0.05; Figure 6.3B), and old SHRs (pEC_{50} = 7.2 ± 0.02, *n* = 2; *p*>0.05; Figure 6.3C).

6.3.3 Effect of COX inhibition on the dilatation to noradrenaline and forskolin

In the presence of the α_1-adrenoceptor antagonist prazosin (1 μM), L-NAME and U46619-induced tone, noradrenaline (NA) applied in the bath concentration-dependently dilatated arteries of young Wistar rats (pEC_{50} = 6.11 ± 0.06, *n* = 8; Figure 6.4A). The dilatation was not significantly augmented by indomethacin (*n* = 4; *p*>0.05) or endothelium denudation (*n* = 8; *p*>0.05). When NA was perfused through the lumen, indomethacin slightly enhanced the response (from E_{max} = 89.8 ± 6.5%, pEC_{50} = 5.68 ± 0.04, *n* = 6, to E_{max} = 99.1 ± 0.9%, pEC_{50} = 6.01 ± 0.02, *n* = 2; *p*>0.05; Figure 6.4B).

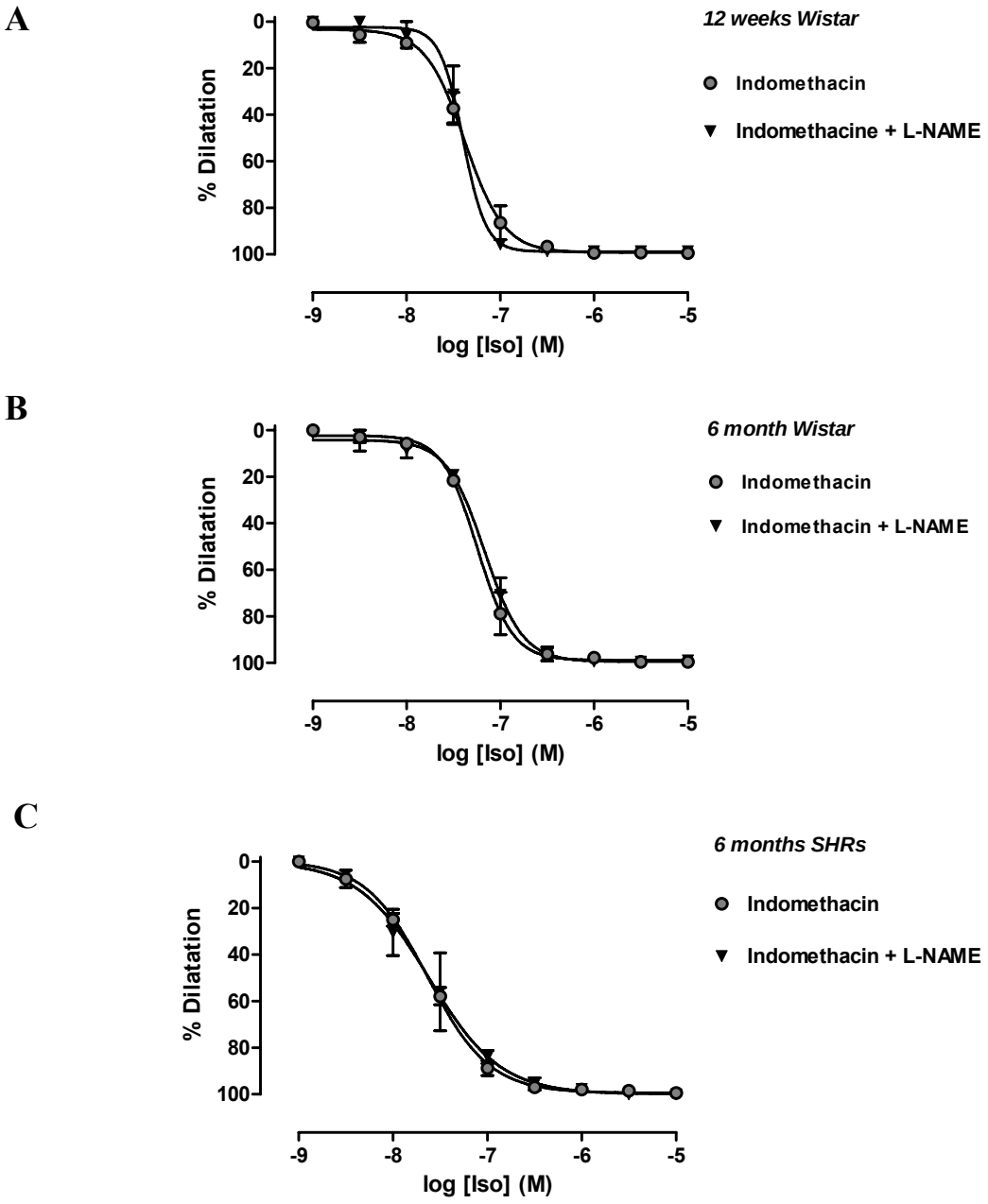


Figure 6.3 Effect of L-NAME on the concentration-dependent dilatation to isoprenaline of rat pressurized mesenteric arteries in the presence of indomethacin

A. Dilatation to isoprenaline in arteries from 12 week old Wistar rats was not affected by L-NAME (100 μM) in the presence of indomethacin (10 μM; n = 2-7).
B. L-NAME did not modify the dilatation to isoprenaline of arteries from 6 months old Wistar rats (n = 4).
C. L-NAME also failed to inhibit the dilatation of arteries from 6 month old SHRs (n = 2).
Results shown are the mean ± s.e.mean.

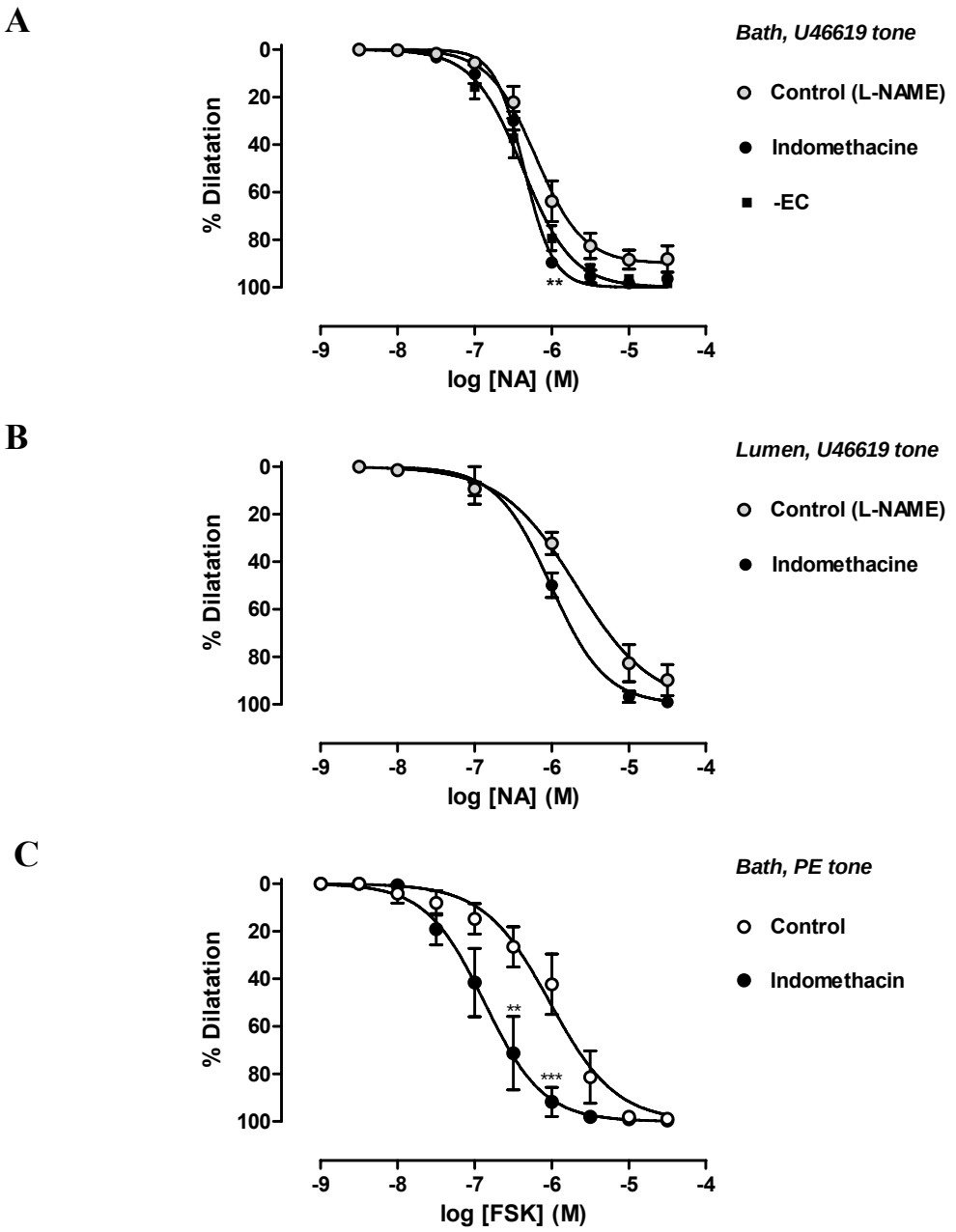


Figure 6.4 Effect of indomethacin on the concentration-dependent dilatation to NA and forskolin in PE-preconstricted pressurized small mesenteric arteries from 12 week old Wistar rat

A. In the presence of prazosin (1 μM) and U46619-induced tone, abluminal application of noradrenaline (NA) revealed dilatation, which was only slightly enhanced by indomethacin (10 μM) or endothelium denudation (n = 4-9).

B. Dilatation to luminal perfusion of NA was moderately augmented by indomethacin (n = 2-6).

C. The adenylyl cyclase activator forskolin (FSK) induced dilatation, which was significantly augmented by indomethacin (n = 4, p<0.05).

Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01, ***p<0.001 vs. control.

On the other hand, dilatation to the adenylyl cyclase activator forskolin (FSK) of PE-preconstricted arteries was significantly enhanced by indomethacin (from pEC_{50} = 6.02 ± 0.06, to pEC_{50} = 6.87 ± 0.02, n = 4; p<0.05; Figure 6.4C).

6.3.4 The effect of the agonist used to evoke the tone on the dilatation to isoprenaline of rat pressurized small mesenteric arteries

Dilatation to isoprenaline of arteries from young Wistar rats was dependent on the agonist used to evoke the tone. When thromboxane mimetic U46619 was used, it significantly shifted the isoprenaline CRC to the right in comparison to PE-induced tone (from pEC_{50} = 7.35 ± 0.06, n = 4, to pEC_{50} = 6.66 ± 0.1, n = 6; p<0.05; Figure 6.5A); similar to those obtained in response to adrenaline and NA with prazosin present (Figure 6.5B). Preconstriction with U46619 suppressed the dilatation to isoprenaline of the arteries from old Wistar rats to a greater extent (E_{max} = 47.6 ± 29.2%, n = 4). Indomethacin did not modify significantly the dilatation of the arteries from young rats preconstricted with U46619 (to pEC_{50} = 7.1 ± 0.2, n = 4). In contrast, it restored the dilatation in arteries from old rats preconstricted with U46619, back to a similar level observed in the younger rats (E_{max} = 97.6 ± 2.4, pEC_{50} = 7.16 ± 0.16, n = 3).

6.3.5 The effect of the agonist used to induce the tone on the conducted dilatation

Luminal perfusion of ACh (1 μM) or isoprenaline (1 μM) into a Branch 1 of a triple-cannulated artery induced a pronounced local dilatation, that spread upstream into the Feed branch. Preconstriction with U46619 markedly enhanced the magnitude of spreading for both ACh (from 2.42 ± 1% to 35.36 ± 9.1% at 3 mm upstream, n = 8-21) and isoprenaline (from 1.91 ± 2.9% to 35.06 ± 9.4% at 3 mm upstream, n = 6-9) in comparison to when PE was used to induce tone.

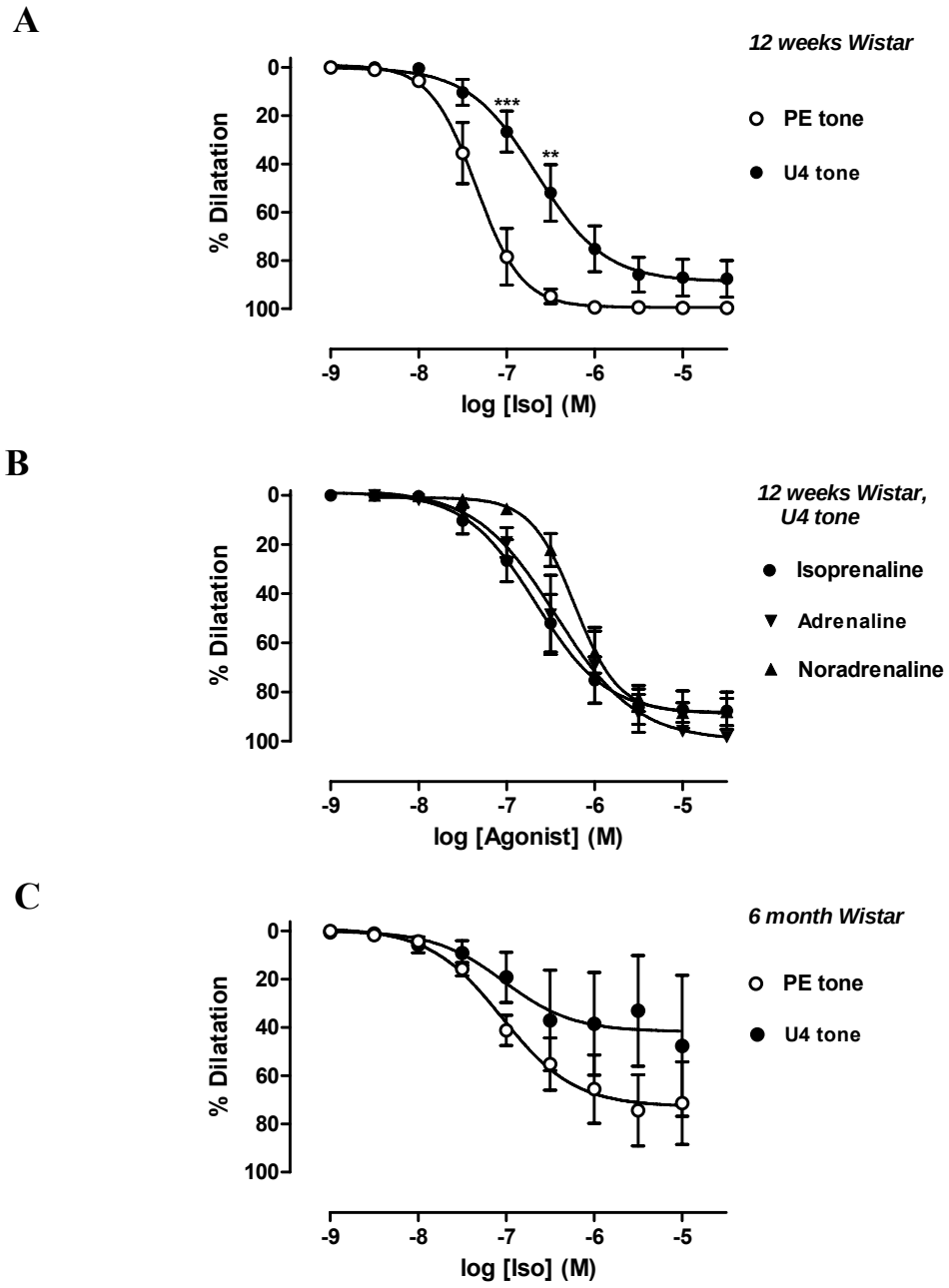
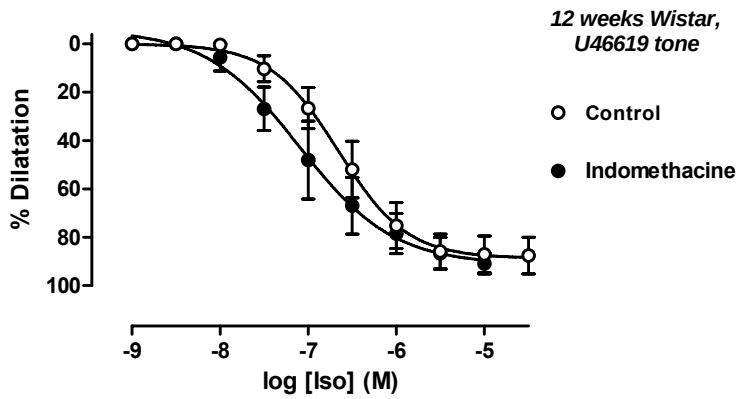


Figure 6.5 Effect of agonist used to evoke the tone on the concentration-dependent dilatation to isoprenaline of rat pressurized small mesenteric arteries

A. Preconstriction of the arteries from 12 week old Wistar rats with U46619 attenuated the dilatation to isoprenaline in comparison with the preconstriction with PE (n = 4-6).
B. When compared to the dilatations evoked by adrenaline and NA, dilatation of the U46619 preconstricted arteries to isoprenaline was similar (12 weeks Wistar rats, n = 4-8).
C. Preconstriction with U46619 attenuated the dilatation to isoprenaline of the arteries from 6 month old Wistar rats even more (n = 4).
Results shown are the mean ± s.e.mean; ****p<0.01, ***p<0.001 vs. PE.

A



B

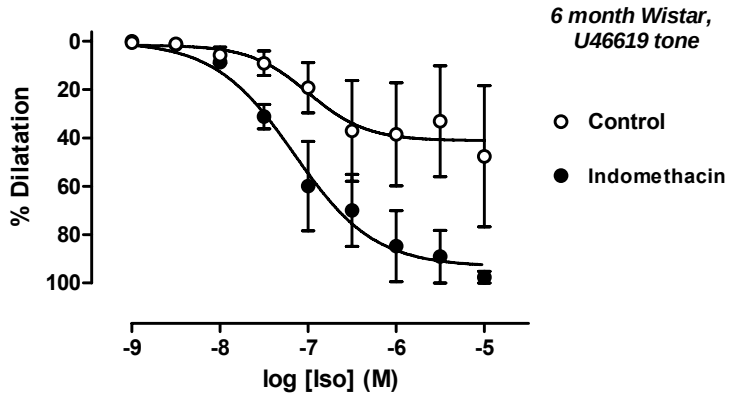
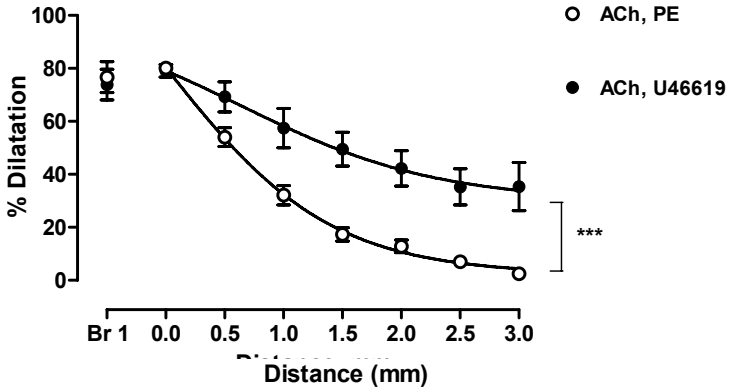


Figure 6.6 Effect of indomethacin on the concentration-dependent dilatation to isoprenaline of the U46619-preconstricted rat pressurized small mesenteric arteries

A. Indomethacin (10 μM) did not affect the isoprenaline-induced dilatation in arteries from 12 week old Wistar rats (n = 4-6).
B. Indomethacin restored the isoprenaline-mediated dilatation of the arteries from 6 month old Wistar rats to levels observed in younger rats (n = 3-4).
Results shown are the mean ± s.e.mean.

A



B

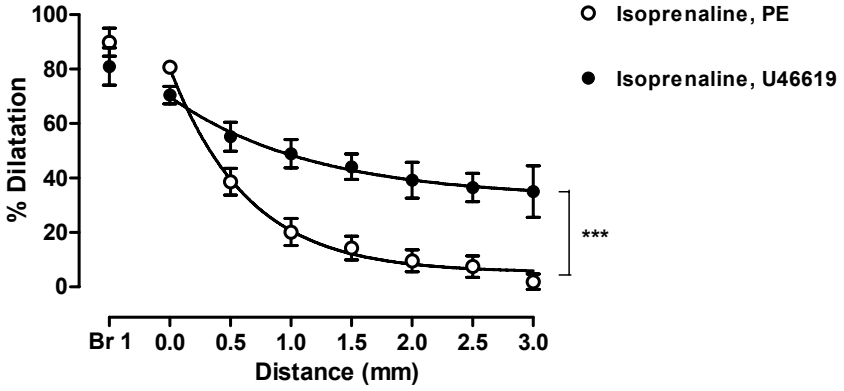


Figure 6.7 Effect of the agonist used to evoke tone on the conducted dilatation in triple-cannulated small mesenteric arteries from 12 week old Wistar rats

A. Summarized data illustrating the conducted dilatation responses to luminal application of ACh (1 μM) in arteries preconstricted with U46619 (n = 8) or PE (n = 21).
B. Comparison of conducted dilatation responses to luminal application of isoprenaline (1 μM) in arteries preconstricted with U46619 (n = 8) and PE (n = 9).
Results shown are the mean ± s.e.mean; ***p<0.001 vs. PE.

6.4 Discussion

It is well known that vascular function is impaired during essential hypertension and aging. Our results contribute to and extend this knowledge through a clear demonstration that attenuated vasodilatation to endothelium-dependent and -independent stimuli in old and diseased resistance vessels is attributed to COX signaling.

All major discoveries on the subject of endothelium-derived contracting factors (EDCFs) in age and hypertension have predominantly been obtained in conductive vessels, mainly aortas from normotensive and hypertensive rats, using the endothelium-dependent agonist ACh (Feletou *et al.*, 2009). Indeed, COX metabolites were shown to significantly attenuate dilatation to ACh of aortas from old (Matz *et al.*, 2000) or hypertensive rats (Sekiguchi *et al.*, 2001). However, no effect of nonselective COX inhibitor indomethacin was present in our experiments performed on resistance mesenteric arteries from young and old normotensive rats. A modest impairment of the ACh response due to COX signalling could be seen only in the 6 month old SHRs, which supports the evidence that COX metabolites are the EDCFs in this rat model (Feletou *et al.*, 2009).

In contrast, endothelium-independent dilatation evoked by β-adrenoceptor agonist displayed a marked reduction in mesenteric arteries from old, and especially from old hypertensive rats. This data correlates with several previous publications. Impaired dilatation of rat aortae or mesenteric arteries to isoprenaline was shown in old rats (Borkowski *et al.*, 1992; Gomez *et al.*, 2008; Kang *et al.*, 2007; Tsujimoto *et al.*, 1986) and aorta from young SHRs (Borkowski *et al.*, 1992; Gomez *et al.*, 2008), whilst relaxation of the mesenteric arteries from young SHRs were shown to have no significant difference in comparison to the control normotensive rats (Blankesteijn *et*

al., 1996). The effect of aging on the isoprenaline-induced dilatation in mesenteric arteries from SHRs was not yet described, however, a reduced hyperpolarization was shown for both young (Goto *et al.*, 2001) and old (Fujii *et al.*, 1999) animal groups. Therefore, it seems possible that impairment of β-adrenoceptor responsiveness of small resistance arteries contributes to the age and hypertension-related blood pressure elevations to a comparable, if not greater extend than the blunted endothelial cholinergic receptor signalling.

Whilst having a very modest effect on the responses to β-adrenergic agonists in young normotensive rats, indomethacin dramatically improved the dilatation to isoprenaline in old and hypertensive mesenteric arteries, revealing a similar response that was observed in young normotensive animals. These results do not suport the idea that impaired receptor density, G-protein coupling (Gurdal *et al.*, 1995), cAMP synthesis or PKA function (Deisher *et al.*, 1989) are responsible for the reduced response to isoprenaline in age or hypertension, but supports the evidence that COX may participate in the attenuation of the relaxation to isoprenaline, which was established in aortae from old rats (Kang *et al.*, 2007).

Removal of the endothelium was shown to augment the endothelium-independent dilatation (to isoprenaline, NO donor, 8-bromo-cGMP, calcitonin gene-related peptide) in mesenteric arteries from young rats (Iwatani *et al.*, 2008) as well as decrease prostanoid levels in aortae from old rats (Kang *et al.*, 2007). In our experiments, the dilatation to β-adrenergic agonists of young and old arteries was also improved by endothelium denudation, reflecting the possibility of a contractile factor released by the endothelium. This may reflect a role for endothelial β-adrenoceptors, which were shown to be expressed in the intima, but apparently did not participate in the relaxation to isoprenaline (Briones *et al.*, 2005a). On the other hand, it is also possible that cAMP

spreads to adjacent endothelial cells from the smooth muscle cells through the gap junctions, as was shown for Rat-1 cells (Ponsioen *et al.*, 2007).

It is not known whether COX is basally releasing prostanoids or is stimulated by adrenergic agonists, but the fact that the dilatation to isoprenaline was much more attenuted than to ACh, leads us to suggest that isoprenaline may enhance COX signaling. Endothelial COX is known to be activated by a rise in $[Ca^{2+}]_i$ (Tang *et al.*, 2007), but how β-adrenergic signaling can stimulate endothelial cell COX is not clear. There are several lines of evidence that the cAMP-PKA pathway may enhance COX signalling. First, extracellular Ca^{2+} can mediate PKA-dependent expression of COX-2 in osteoblasts (Choudhary et al., 2004). Secondly, in renal tissue the effect of the PKA pathway on expression of COX-2 has also been demonstrated (Steinert et al., 2009). Further, in bovine aortic endothelial cells, a rise in cAMP up-regulates COX-2 in a PKA-dependent manner (Samokovlisky *et al.*, 1999). Finally, another pathway may provide stimulation of phospholipase A by β-adrenoceptor, leading to production of arachidonic acid (Borda *et al.*, 1998; Neuman *et al.*, 2002), a precursor for COX metabolites.

COX products may underlie a range of pathophysiological processes in the endothelium (Feletou *et al.*, 2010a; Wong *et al.*, 2010b). One of the pathways may be revealed from the observation that COX signalling or TP receptor activation triggers an impairment of eNOS (Bratz *et al.*, 2004; Liu *et al.*, 2009) or guanylyl cyclase function (Arshad *et al.*, 2006). Despite some previous publications (Figueroa *et al.*, 2009b; Graves *et al.*, 1993), we did not see any eNOS-dependent component to the β-adrenoceptor-mediated dilatation (see Chapter 4). With the knowledge that β-adrenergic signaling may stimulate COX, it seemed therefore possible that this enzyme may interfere with a NO-dependent component in response to β-adrenoceptor agonists. If

this was the case, indomethacin should reveal an eNOS-dependent dilatation. However, in our experiments L-NAME still had no effect in all young, old, and hypertensive animals, implying any lack of or a minimal role for eNOS in the vasodilatation to β-adrenoceptor stimulation, even after COX was inhibited.

Many prostanoids are able to bind TP receptors, activation of which is a key event in triggering cardiovascular pathogenesis (Feletou *et al.*, 2010a; Feletou *et al.*, 2009). However, how cross-talk between TP and β-adrenoceptor occurs is not defined.

U46619 is often used as a contractile agent, especially when α-adrenoceptors are inhibited to reveal a β-adrenergic component of the response to NA/adrenaline (Filippi *et al.*, 2001; Garland *et al.*, 2010b; Macdonald *et al.*, 1987). β-adrenoceptors are coupled to G_s proteins, which stimulate adenylyl cyclase, leading to vasodilatation through activity of cAMP-dependent enzymes. The concentration of cAMP is regulated by phosphodiesterases, (Boswell-Smith *et al.*, 2006). In rat carotid artery, phosphodiesterases were shown to be stimulated by U46619 (Liu *et al.*, 2010), suppressing dilatation to isoprenaline. In our experiments, treatment with U46619 also resulted in reduced dilatation to β-adrenergic stimulation, indicating that a similar pathway may be involved.

On the other hand, coupling of TP receptor to PKC (Budzyn *et al.*, 2006) may lead to a PKC-dependent reduction of K_{ATP} channel function in rat mesenteric myocytes (Kubo *et al.*, 1997). Although this channel does not play a significant role in the local dilatation to β-adrenoceptor agonists (Garland *et al.*, 2011; White *et al.*, 2001), it may potentiate a reduction in dilatation when activated together with the inhibitory mechanism described above (Liu *et al.*, 2010).

Importantly, TP receptors by themselves can lead to release of prostanoids from cultured endothelial cells and mesenteric arteries (Bolla *et al.*, 2004; Hunt *et al.*, 1992;

Nicholson *et al.*, 1984). This may create a negative loop: β-adrenoceptor signalling promotes release of prostanoids, which in turn can activate TP receptors to evoke the release of more prostanoids and facilitate cAMP breakdown leading to suppression of β-adrenoceptor signalling. This pathway may be particulary prevelant in pathophysiological conditions, explaining why indomethacin dramatically improved the dilatation to isoprenaline in U46619-preconstricted arteries from old rats in our experiments.

Despite having a pronounced inhibitory action on the local dilatation, precontraction with the TP receptor agonist improved the conducted dilatation to both ACh and isoprenaline, when compared to the PE-induced tone. NA, acting on α-adrenoceptors, was also shown to reduce conducted vasodilatation in hamster feed arteries (Haug *et al.*, 2003). The reasons for this may relate to the signalling pathways activated by these two receptors. Both activate $G_{q/11}$ protein, which is coupled to phospholipase C, resulting in breakdown of PIP_2 and production of DAG, the endogenous activator of PKC. Generally, PKC phosphorylates connexin 43 and reduces the intercellular communication via gap junctions (Bao *et al.*, 2007; Heyman *et al.*, 2009; Inoguchi *et al.*, 1995); however, recent research demonstrated that in cultured cardiomyocytes PKC activation enhanced dye transfer through enhanced gap junctional permeability (Duquesnes *et al.*, 2010).

In contrast, TP receptors are also coupled to G-proteins of the $G_{12/13}$ family, and the constriction to U46619, in comparison to PE, relies more on the sensitization mechanism through Rho kinase, so dependence on rises in $[Ca^{2+}]_i$ is less (Shaw *et al.*, 2004). This by itself may lead to reduced PKC activity and reduced connexin phosphorylation, that together with Rho A -dependent enhancement of gap junctional coupling (Derangeon *et al.*, 2008) may increase the spread. Additionally, lack of $[Ca^{2+}]_i$

may result in closure of BK_{Ca} channels, and, thereby, dissipation of the hyperpolarizing current that normally reduces the conducted dilatation in the presence of α_1-adrenoceptor agonist (Beleznai *et al.*, 2011).

Another explanation may arise from the observation that, in comparison to NA, U46619-evoked constriction was accompanied by a smaller depolarization (Plane *et al.*, 1996). This may mean a smaller increase in membrane potential is effective in mediating closure of L-type Ca^{2+} channels and thus evoking subsequent relaxation. As a result, the negative charge produced by stimulated cells dissipates to a lesser extent and can reach greater numbers of adjacent cells.

Taken together, these results demonstrate that stimulation of β-adrenergic receptors is associated with the activation of COX, linked to an inhibitory action on vasodilatation, especially in old and hypertensive animals. Endothelial denudation enhances dilatation to a smilar extent as treatment with COX inhibitors. The enzyme activity is not implicated in absence of the L-NAME–sensitive component of the dilatation to β-adrenergic agonists. The products of COX may act on TP receptors, to suppress the local dilatation to β-adrenergic agonists, whilst in some way enhancing conducted dilatation

Acknowledgments

I would like to thank Dr. Claire Hamill for her kind help with the animal maintenance and her interest and support during this project, and Dr. Robin Hiley for his useful advice and for providing a recipe for indomethacin preparation and usage.

Chapter 7. Nitroxyl anion evokes local and conducted dilatation in rat mesenteric resistance arteries; a role for K^+ channels

7.1 Introduction

The endothelium participates in the regulation of vascular tone by means of electrical coupling via gap junctions and release of various vasoactive agonists and gaseous molecules. One of the gaseous molecules, NO, plays a particularly important role in vascular physiology (Furchgott *et al.*, 1991; Moncada *et al.*, 2006).

Activation of the endothelium is usually associated with a rise in $[Ca^{2+}]_i$ that stimulates eNOS, leading to the release of NO by the mechanisms described in Section 1.3.2. NO can exist in several different redox forms, among which uncharged free radical state of the NO molecule (nitrogen monoxide, $NO^{\bullet}$) and nitroxyl anion (NO^-) are present (Hughes, 1999). $NO^{\bullet}$ has been considered to be the main participant in vasodilatation responses to different NO donors or after stimulation of endothelium (Dierks *et al.*, 1996a; Feelisch, 1993). However, endothelium-dependent relaxation of rodent aortas, which mainly goes through NO pathway, was demonstrated to be sensitive to both, $NO^{\bullet}$ and NO^- scavengers (Ellis *et al.*, 2000; Wanstall *et al.*, 2001).

It has been shown that NO^- can be produced by NOS in the absence of BH_4 (Figure 7.1). Electron donation from BH_4 to the heme-bound O_2 enables it to be an electron acceptor later, during L-arginine oxidation, promoting formation of a ferric heme-NO complex, thus ensuring that the enzyme generates $NO^{\bullet}$ instead of NO^{-}. However, when BH_4 is not available, a ferrous heme-NO complex builds up, resulting in formation of NO^- (Adak *et al.*, 2000). An additional way of NO^- formation is by reduction from $NO^{\bullet}$ by mitochondrial cytochrome C (Sharpe *et al.*, 1998). Moreover, it has been shown that the initial product of eNOS may be not $NO^{\bullet}$, but NO^- (Schmidt *et al.*, 1996), which could be further converted into $NO^{\bullet}$ by means of superoxide dismutase (Fukuto *et al.*, 1993; Hobbs *et al.*, 1994; Schmidt *et al.*, 1996), whilst a backward conversion of $NO^{\bullet}$ to NO^- is highly unfavourable (Bartberger *et al.*, 2002).

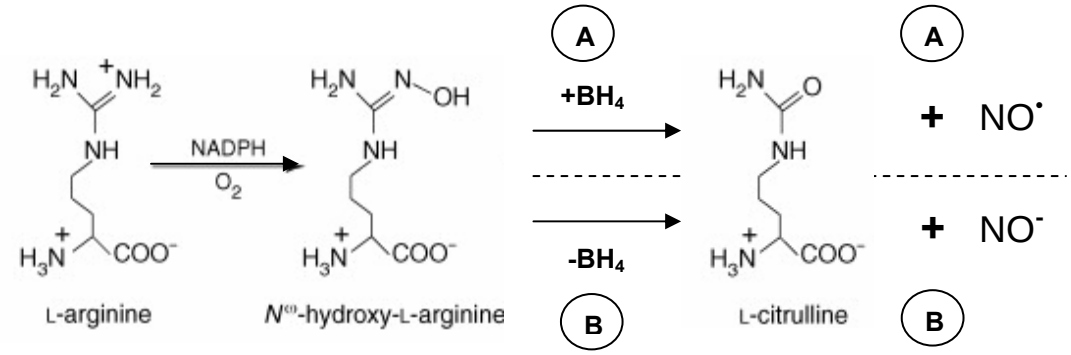


Figure 7.1 Diagram illustrating the pathway of possible endogenous NO^- production by NOS

The heme can react with L-arginine in the presence or absence of BH_4. If BH_4 is present, it provides electron for the heme and, at a later point, the reduced BH_4 can accept the electron from heme-N-hydroxy-L-arginine complex, ensuring formation of the $NO^\bullet$ (path A). However, if the reductase domain provides the electron for the heme, then the reaction can only generate NO^- (path B) (Adak *et al.*, 2000; Irvine *et al.*, 2008).

Under physiological conditions NO^- exists as a conjugated weak acid, HNO (nitroxyl). As a donor of nitroxyl species researchers widely use Angeli's salt (sodium trioxodinitrate, $Na_2N_2O_3$), which, after being dissolved in aqueous solution at physiological pH, decomposes according to the following equation (Amatore *et al.*, 2007):

$$HN_2O_3^- \rightarrow HNO + NO_2^-$$

NO-gassed solution is generally used as a source of $NO^\bullet$ (Favaloro *et al.*, 2009; Rajanayagam *et al.*, 1993; Wanstall *et al.*, 2001), whilst other NO donors, such as sodium nitroprusside can produce both, $NO^\bullet$ and NO^- (Irvine *et al.*, 2003a).

By means of pharmacological tools it became possible to distinguish between the two main forms of NO. $NO^\bullet$ can be inhibited by a free radical scavengers such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (Irvine *et al.*, 2003a; Li *et al.*, 1999b) or hydroxocobalamin (OHCbl) (La *et al.*, 1996; Li *et al.*, 1999a; Rajanayagam *et al.*, 1993), whilst L-cysteine in millimolar concentrations is

widely used to scavenge NO^- (Arvola *et al.*, 1992; Ellis *et al.*, 2000; Irvine *et al.*, 2007; Pino *et al.*, 1994a; Wanstall *et al.*, 2001).

In rodent conduit vessels such as aorta (Irvine *et al.*, 2007; Wanstall *et al.*, 2001), and resistance arteries such as mesenteric artery (Andrews *et al.*, 2009; Favaloro *et al.*, 2007; Irvine *et al.*, 2003b), the HNO donor Angeli's salt was capable to evoke functional responses, similar to $NO^{\bullet}$, via activation of soluble guanylyl cyclase. Moreover, NO^- was reported to be endogenously generated in response to ACh or ATP in sufficient amounts to evoke relaxation of rat arteries (Andrews *et al.*, 2009; Ellis *et al.*, 2000).

However, NO^- was found to have even wider target area in the cardiovasculature than $NO^{\bullet}$ (Irvine *et al.*, 2008; Martin, 2009). This includes direct activations at ryanodine receptors and SERCA (Tocchetti *et al.*, 2007), increases in the myofilament sensitivity to Ca^{2+} by modulation of myofilament proteins that harbour reactive thiolate groups (Dai *et al.*, 2007), lack of development of vascular tolerance to NO^- (Irvine *et al.*, 2007; Irvine *et al.*, 2008), and robust hyperpolarization of smooth muscle (Andrews *et al.*, 2009; Favaloro *et al.*, 2009). The latter, which does not occur in response to $NO^{\bullet}$, is achieved by activation of K_v channels (Andrews *et al.*, 2009; Favaloro *et al.*, 2009).

All previous studies of effects of NO^- in rat mesenteric arteries were performed by means of wire myography (Andrews *et al.*, 2009; Favaloro *et al.*, 2007; Irvine *et al.*, 2003b), and whether the results will be confirmed in more physiological conditions is unknown. Additionally, hyperpolarizing stimuli can evoke conducted vasodilatation (Dora, 2010; Garland *et al.*, 2011; Takano *et al.*, 2004; Winter *et al.*, 2007), and if NO^- can hyperpolarize vascular smooth muscle, then it may evoke the conducted dilatation. Therefore, the aim of this study was to investigate the effects of NO^- in the local and conducted dilatation in rat pressurized mesenteric arteries.

7.2 Methods

7.2.1 Rat mesenteric artery isolation and preparation

See Section 2.1 for methods of artery isolation and preparation.

7.2.2 Pressure myography

Arteries were cannulated and pressurized to 70 mmHg as described in Section 2.2. Pressure myography was used to study both local and conducted responses.

7.2.3 Measurement of local responses

Local dilatation responses were measured using double cannulated vessels, as described in the Section 2.2.2. Vessels were submaximally preconstricted with PE, then raising concentrations of studied agonist cumulatively or non-cumulatively were applied in bath (abluminally). Vasomotion was described as synchronized, if the contraction-dilatation cycles followed the shape of sigmoid curve with a near equal sizes of the pikes and distances between the pikes.

7.2.4 Measurement of conducted dilatation responses

Experiments for conducted dilatation studies were performed using triple cannulated vessels. Agonist together with carboxyfluorescein was perfused via side branch (Branch 1), as detailed in section 2.2.3..

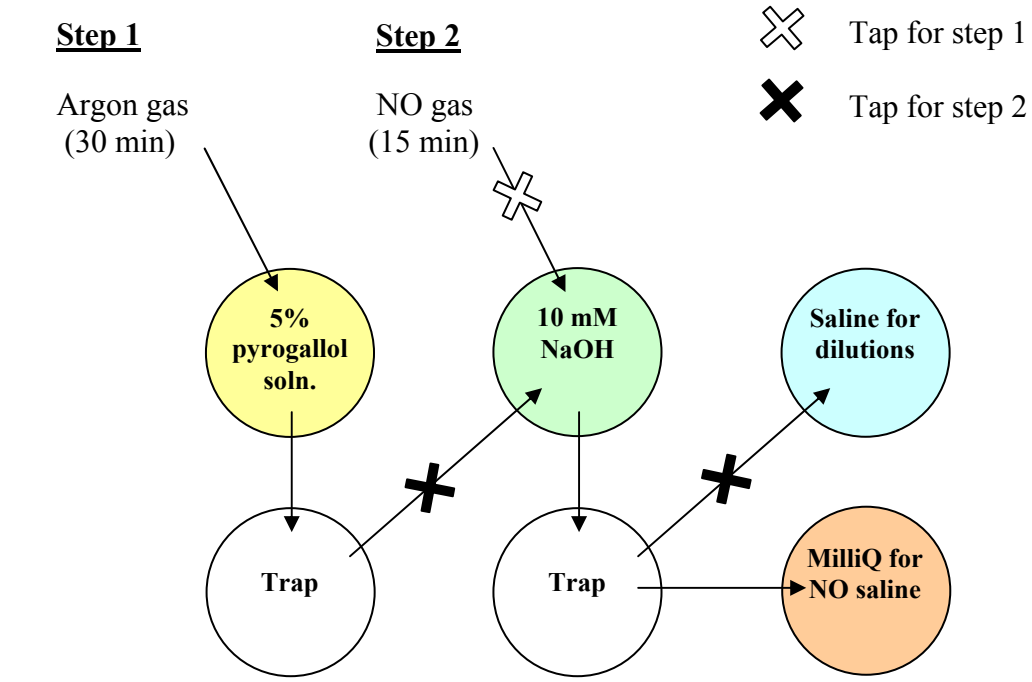
7.2.5 Data analysis

Data were collected and analyzed as described in Section 2.5.

7.2.6 Drugs and solutions

In these experiments, normal MOPS-buffered solution was used, except of L-cysteine was added into a specially prepared MOPS-buffered solution with a reduced amount of NaCl to balance the excess of Na^+ incoming with NaOH during adjustment of pH (see the method below). In order to prevent decomposition, HNO donor, Angeli's salt, (sodium trioxodinitrate) was dissolved in 10^{-2} M of NaOH. In aqueous solutions, Angeli's salt exists in a protonated form HNO, and decomposition of 1 M of Angeli's salt gives 0.54 M of NO^- (Irvine *et al.*, 2008). In order to obtain a saturated solution of $NO^{\bullet}$, de-gassed MOPS-buffered solution was bubbled with NO gas for 15 min, with the final NO concentration of 1.94 mM (Zacharia *et al.*, 2005) (Figure 7.2). pH of solutions containing 4-AP (150 μM) and TEA (1 mM) was additionally adjusted to 7.4±0.2. All other drugs and solutions were prepared as detailed previously (see section 2.6).

<u>L-cysteine MOPS buffer contains</u> (in mM): NaCl, 135; KCl, 4.7; $CaCl_2$, 2.0; $MgSO_4 \cdot 7H_2O$, 1.17; MOPS, 2.0; $NaH_2PO_4 \cdot H_2O$, 1.2; glucose, 5.0; pyruvate, 2.0; EDTA, 0.02; NaOH, ~12.75; L-cysteine, 3; with pH adjusted to 7.40 ± 0.02 (at 37°C).



`

Figure 7.2 Scheme illustrating bubbling cascade for saturated NO$^{\bullet}$ solution

Step 1. In order to de-gas solutions, argon gas was first bubbled for 30 minutes through solution of pyrogallol (5%; yellow circle), then was perfused through NaOH solution (10 mM; green circle), MilliQ water for NO saturation (orange circle), and buffered saline for NO stock dilutions (blue circle).
Step 2. NO gas was bubbled through NaOH solution in order to terminate possible higher oxides of NO (such as NO_2, NO_3), then perfused through Milli Q water for 15 minutes. Dilutions of the saturated NO solution (1.94 mM at room temperature) were prepared using de-gassed buffered saline.
Taps were used to restrict gas to certain solutions during step one and step two.

7.3 Results

7.3.1 Effect of NO gassed solution on the arterial tone

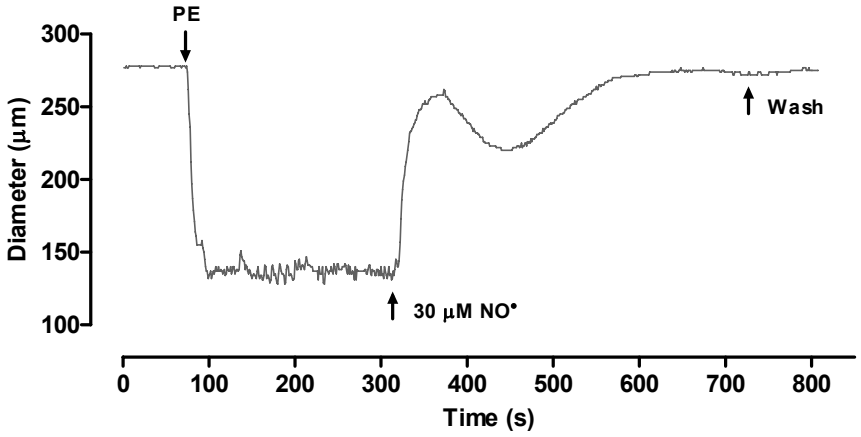
To investigate effects of exogenous $NO^{\bullet}$ on tone of pressurized small mesenteric arteries we used the $NO^{\bullet}$-saturated solution (Zacharia *et al.*, 2005). Application of $NO^{\bullet}$ (0.1 μM - 300 μM of $NO^{\bullet}$) evoked concentration-dependent dilatation of rat small mesenteric arteries (E_{max}=98.2 ± 0.6% at 100 μM, pEC_{50} = 5.3 ± 0.12, n = 7; Figure 7.3A). Whilst low concentrations of $NO^{\bullet}$ led to a transient dilatation, higher concentrations evoked a two-phase response, rapid transient and delayed slow sustained dilatation (Figure 7.3A).

7.3.2 Effect of HNO donor on arterial tone

To establish the effect of NO^{-} on the arterial tone the HNO donor, Angeli's salt, was used in this set of experiments. In PE- preconstricted arteries, bolus application of Angeli's salt (0.1 μM - 300 μM) led to a concentration-dependent dilatation (E_{max}=98.0 ± 0.5% at 100 μM; pEC_{50} = 5.05 ± 0.18; *n* = 3-7), and the degree of the response was similar to the evoked by $NO^{\bullet}$ (p>0.05; Figure 7.4A). Bolus addition of submaximal concentration of Angeli's salt (30 μM) evoked a transient response with a maximum at ~5 minutes, then the magnitude of the response decreased (Figure 7.3B). Additionally, vasomotion induced by PE were stabilized by Angeli's salt administration (30 μM) in 21 cases out of 30.

Since Angeli's salt was diluted in 10 mM NaOH, the vehicle alone was tested, but no significant effect on the arterial tone was observed (p>0.05; *n* = 2; data not shown).

A



B

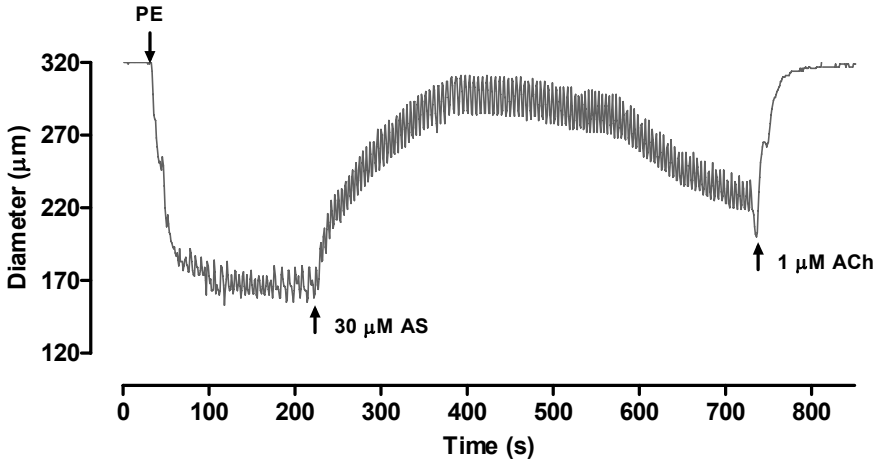
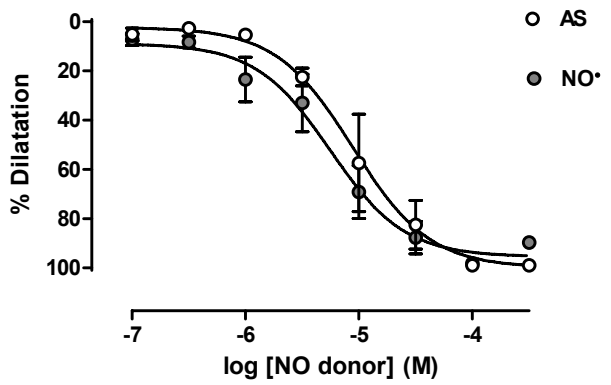


Figure 7.3 Local dilatation to HNO and NO• in small mesenteric arteries

A. Representative trace illustrating dilatation of the artery in response to submaximal concentration of NO• (30 μM) applied as a NO•- saturated solution.
B. Representative trace showing response to submaximal concentration of HNO donor, Angelis salt (AS; 30 μM). ACh (1 μM) was applied at the end of experiment to assess arterial viability.
Time of applications of PE, NO•, AS, and ACh are indicated with arrows.

A



B

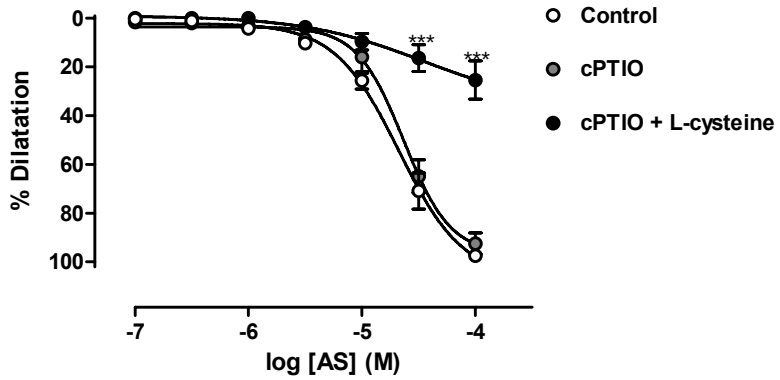


Figure 7.4 Summarized local responses to HNO and NO• of small mesenteric arteries preconstricted with PE

A. Comparison of non-cumulative concentration responses to HNO donor Angelis salt (AS; n = 3-7) and NO•, applied as NO•-saturated solution (n = 7). Values of maximal dilatation to each concentration were used in this figure.
B. Effects of the NO• scavenger carboxy-PTIO (c-PTIO, 200 μM) alone (n = 2) or together with the NO- scavenger L-cysteine (3 mM; n = 6) on the dilatation to cumulative application of AS.

Results shown are the mean ± s.e.mean; ***p<0.001 vs. control.

7.3.3 Effect of L-cysteine on the response to Angeli's salt

In order to ensure that the dilatation to Angeli's salt is mediated by the action of NO^-, but not $NO^\bullet$, effects of the $NO^\bullet$ scavenger, carboxy-PTIO (100 μM), and the NO^- scavenger, L-cysteine (3 μM), on Angeli's salt-induced dilatation were evaluated. Cumulative applications of Angeli's salt evoked complete dilatation of the PE-preconstricted artery in a concentration range from 100 nM to 100 μM (pEC_{50} = 4.7 ± 0.03, *n* = 8). Whilst carboxy-PTIO did not alter the relaxation to Angeli's salt (*n* = 2), L-cysteine, applied together with carboxy-PTIO, markedly inhibited this response (*n* = 6; Figure 7.4B), demonstrating that the dilatation occurs mainly due to NO^- release from Angeli's salt.

7.3.4 Effect of K^+ channel and gyanylyl cyclase inhibition on the responses to Angeli's salt

It was suggested that NO^- predominantly activates K_v channels in vascular smooth muscle and has virtually no effect on BK_{Ca} channel activity (Favaloro *et al.*, 2009; Irvine *et al.*, 2003a). In accordance with this, in our experiment the K_v channel blocker 4-aminopyridine (4-AP, 150 μM) also inhibited the response, however, 4-AP did not block it completely (E_{max} = 49.35 ± 13.4% at 5 min, *n* = 4), also it did not prevent the tone oscillations after precontraction with PE (Figure 7.5B). On the other hand, incubation with the BK_{Ca} inhibitor iberiotoxin (100 nM) evoked contraction of the vessel to 35.3±8.4% of the maximum and vasomotion in four cases of seven (*n* = 7; Figure 7.5C); moreover, both iberiotoxin (*n* = 5) and K_{Ca} channel blocker TEA (1 mM; *n* = 6) significantly suppressed the dilatation to Angeli's salt (E_{max} = 40.8 ± 14.8% and E_{max} = 46.7 ± 14.2% at 5 min, subsequently; $p<0.05$).

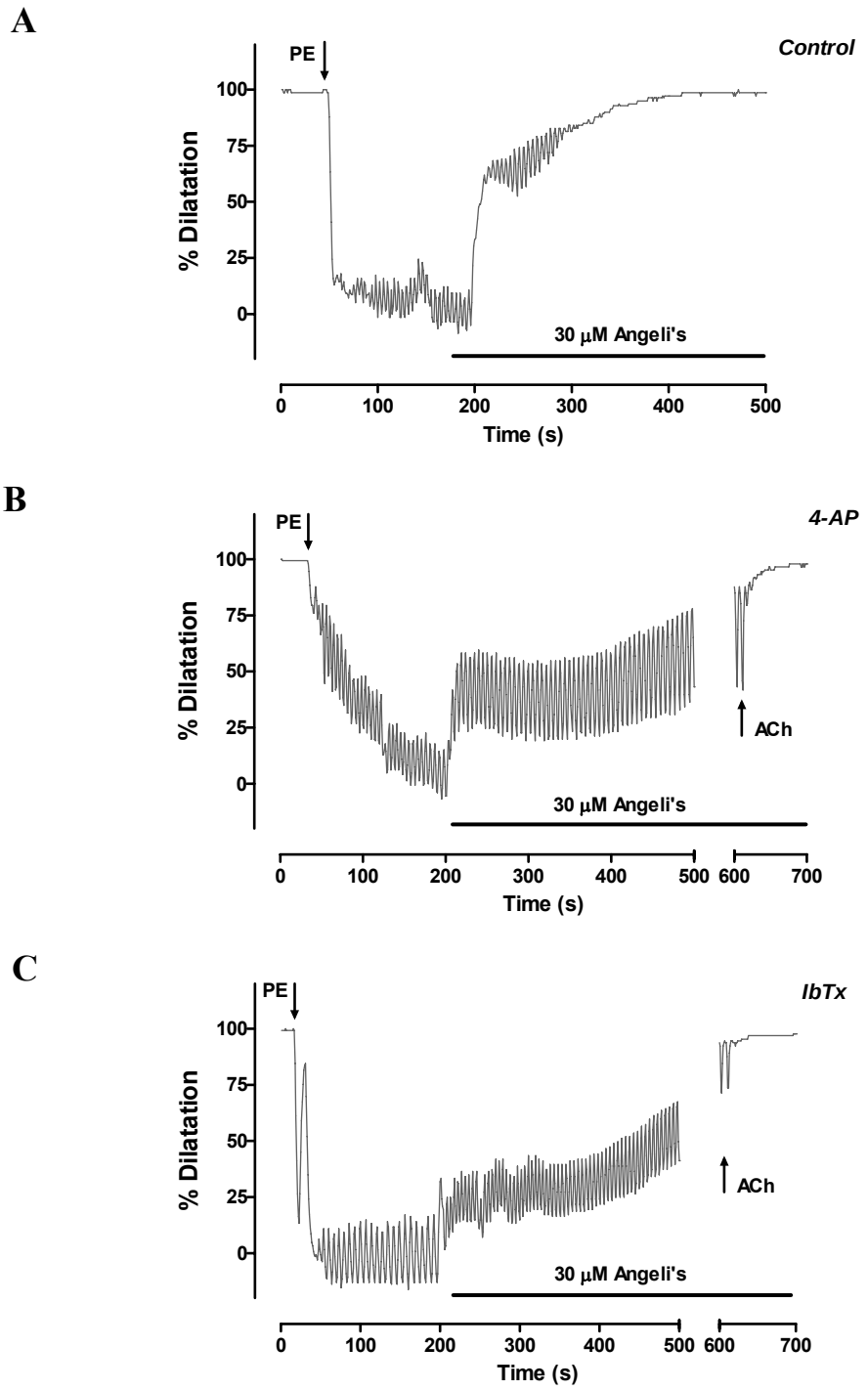


Figure 7.5 Effect of BK_{Ca} and K_v channels inhibition on the local dilatation to Angeli's salt

A. Typical trace illustrating dilatation of the artery to submaximal concentration of the HNO donor Angeli's salt (30 μM) in control.
B. Representative trace showing a response to Angeli's salt in the presence of the K_v channel inhibitor 4-AP (150 μM).
C. Effect of the BK_{Ca} channel inhibitor iberiotoxin (IbTx, 100 nM) on the dilatation to Angeli's salt.
ACh (1 μM) was applied at the end of each trace to test endothelial reactivity.

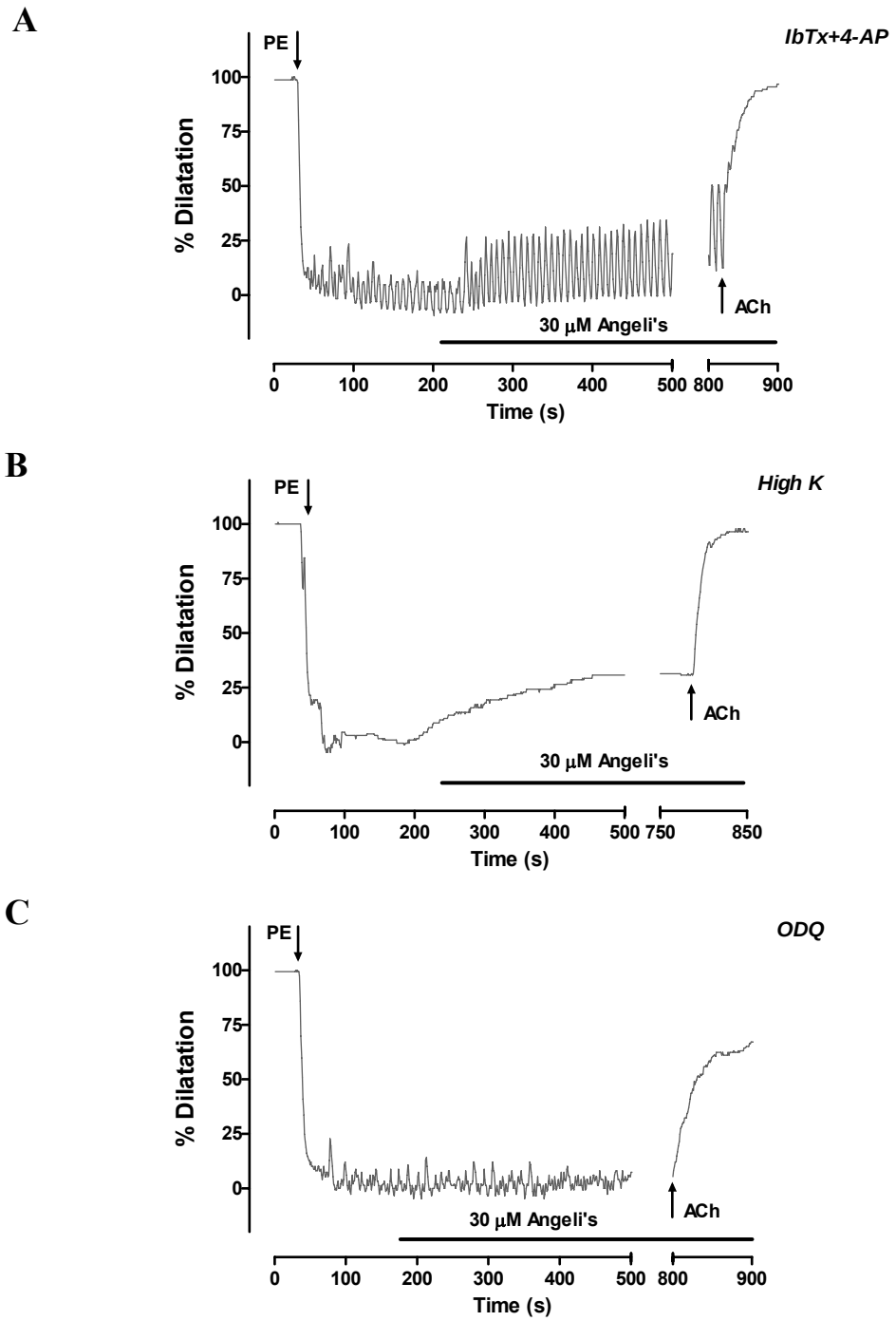


Figure 7.6 Effect of high K^+ and guanylyl cyclase inhibition on the local dilatation to Angeli's salt

A. Representative trace illustrating dilatation of the artery to HNO donor Angeli's salt (30 μM), applied in the presence of the BK_{Ca} channel inhibitor iberiotoxin (IbTx, 100 nM) and the K_v channel inhibitor 4-AP (150 μM).
B. Typical trace showing dilatation to Angeli's salt in the presence of high K (KCl, 45 mM).
C. Original trace demonstrating inhibition of the dilatation to Angeli's salt by the guanylyl cyclase inhibitor ODQ (10 μM).
ACh (1 μM) was applied at the end of each trace to test endothelial reactivity.

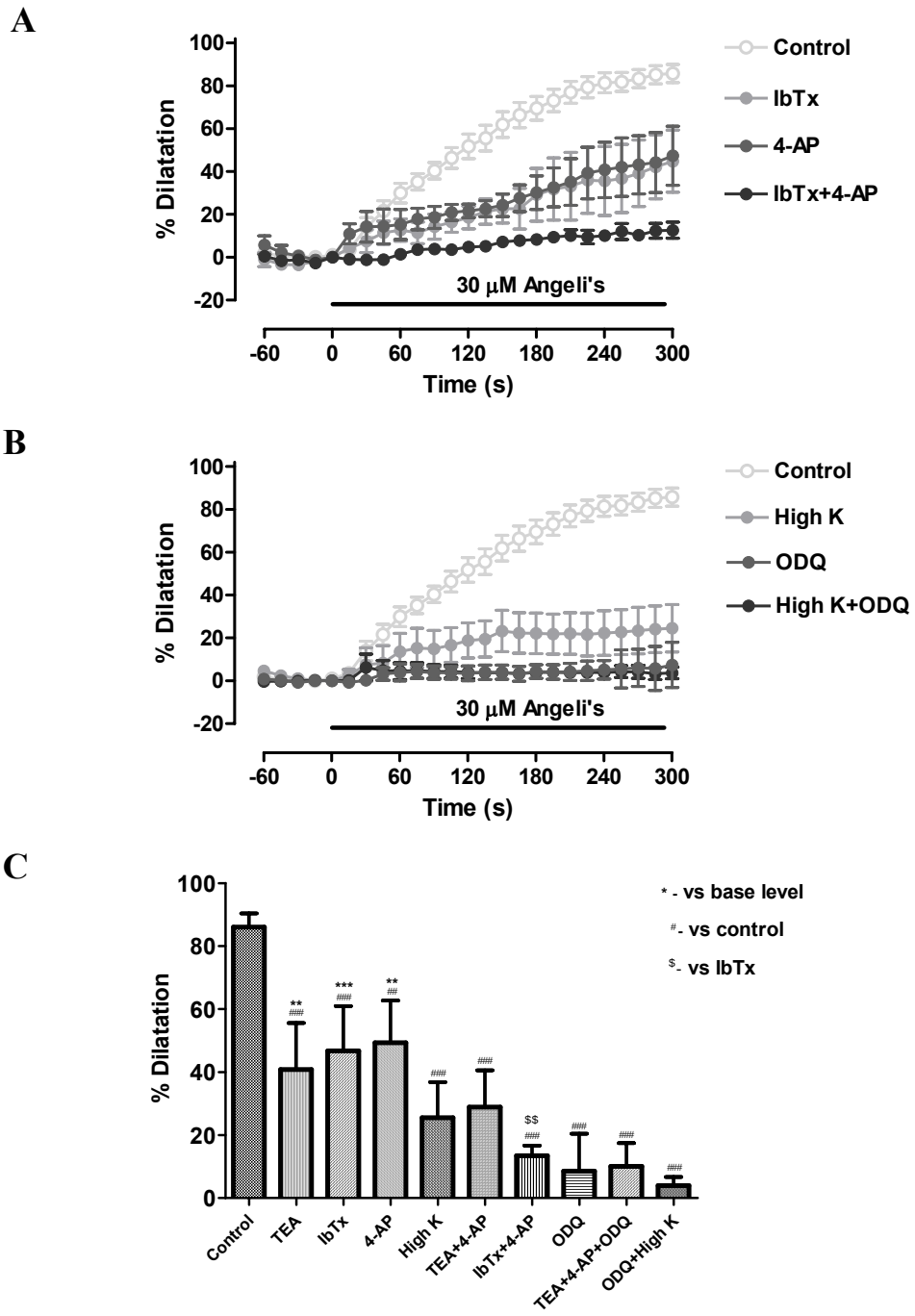


Figure 7.7 Participation of K^+ channels and guanylyl cyclase in the local dilatation to Angeli's salt

A. Averaged data illustrating effects of the BK_{Ca} channel inhibitor iberiotoxin (IbTx, 100 nM; $n = 6$) and the K_v channel inhibitor 4-AP (150 μM; $n = 5$) on the dilatation to the HNO donor Angeli's salt (AS; 30 μM).
B. Effects of high K (45 mM) and the guanylyl cyclase inhibitor ODQ (10 μM; $n = 5$).
C. Summarized data representing effects of IbTx, TEA, 4-AP, high K (KCl, 45 mM) and ODQ on the dilatation to Angeli's salt. Values are taken at 5 min after the Angeli's salt was applied. Results are the mean ± s.e.mean.

Combination of either iberiotoxin or TEA with 4-AP has almost blocked the dilatation (n = 4; Figure 7.7A,C), but failed to prevent vasomotion (Figure 7.6A). High external K^+ (KCl, 45 mM) constricted the vessel (via depolarization) and suppressed both vasomotion and dilatation to Angeli's salt (n = 5; p<0.05; Figure 7.6B and 7.6B,C). The guanylyl cyclase inhibitor ODQ (10 μM) desynchronized vasomotion and abolished the response to the HNO donor (n = 5; p<0.05; Figure 7.6C and 7.7B,C). These data indicate that dilatation to HNO donor is enabled by opening of both K_v and BK_{Ca} channels and depends on guanylyl cyclase activity, whilst vasomotion are sensitive only to high KCl and guanylyl cyclase inhibition.

7.3.5. Role of NO^- in vasomotion observed in PE- preconstricted mesenteric arteries

Since the HNO donor stabilized the vasomotion, whilst $NO^\bullet$ did not, we decided to elucidate the role of endogenous HNO in the coordination of vasomotion. Submaximal preconstriction with PE evoked stable oscillations of tone with a frequency of 0.22 Hz ± 0.01 (n = 15; Figure 7.3B). Scavenging of $NO^\bullet$ with hydroxocobalamin (HXC, 100 μM) resulted in a decrease of synchronization of vasomotion, but did not change the frequency of tone oscillations (0.22 Hz ± 0.01, n = 5; Figure 7.8A). The NO^- scavenger L-cysteine applied luminally and abluminally, markedly suppressed the vasomotion (n = 6; Figure 7.8B). Inhibition of NOS with L-NAME (100 μM) resulted in loss of detectable vasomotion in 7 cases from 12, the rest displayed reduced synchronisation with a small decrease in frequency of tone oscillations (0.16 Hz ± 0.02, n = 5; Figure 7.8C).

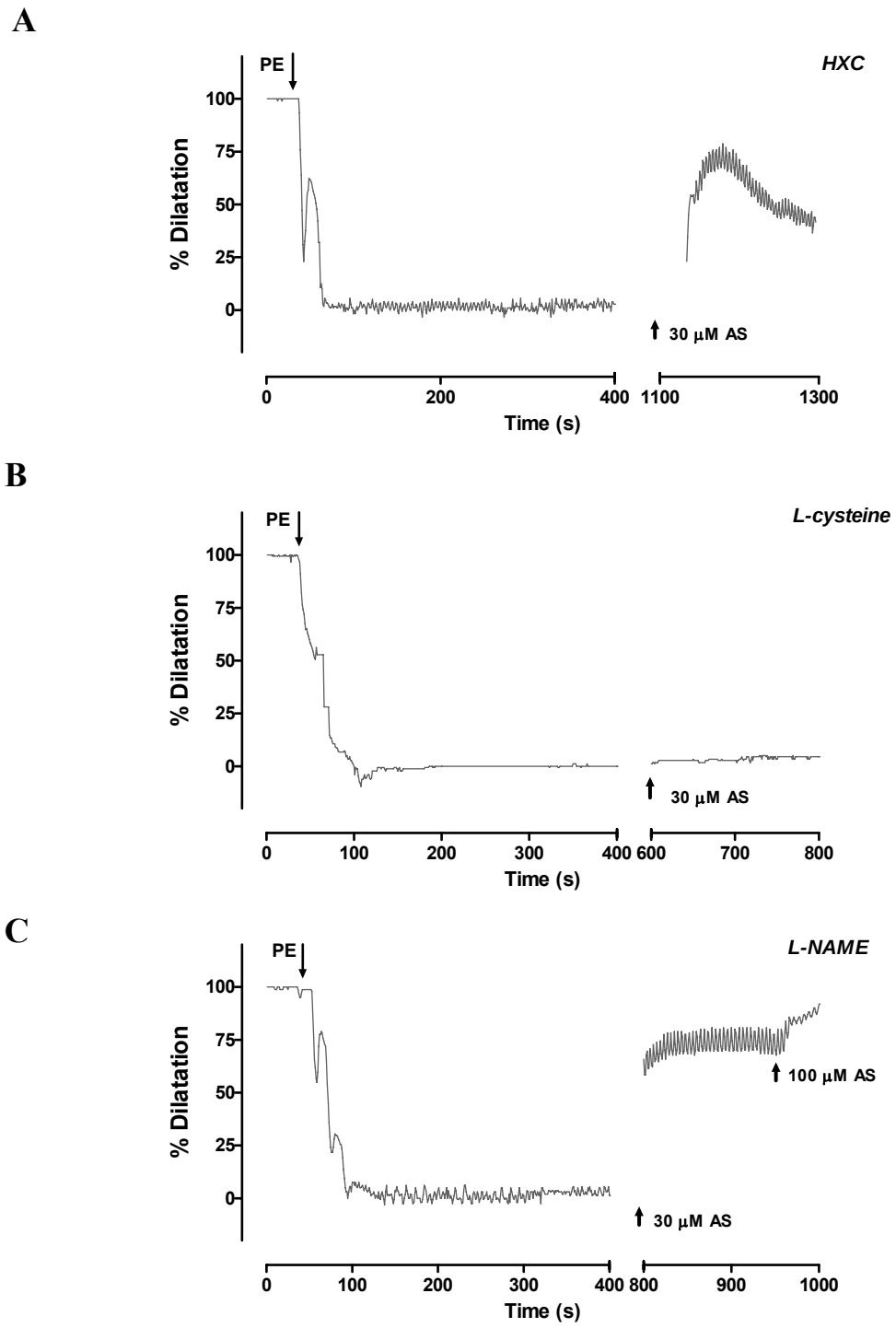


Figure 7.8 Representative traces illustrating effect of endogenous NO on vasomotion in rat small mesenteric arteries preconstricted with PE

A. Typical trace showing vasomotion in the presence of the $NO^{\bullet}$ scavenger hydroxocobalamin (HXC, 100 μM);
B. The NO^{-} scavenger L-cysteine (3 mM) suppresses the oscillations of tone;
C. Inhibition of NOS with L-NAME (100 μM) affects vasomotion.
Application of Angelis salt (30 μM) in the presence of HXC or L-NAME led to the dilatation and synchronization of vasomotion, whilst it failed to evoke dilatation or tone oscillations in the presence of L-cysteine.

7.3.6 HNO donor induces conducted dilatation in the triple-cannulated pressurized mesenteric arteries

It has been earlier demonstrated that hyperpolarizing agents, such as the K_{ATP} channel opener levcromakalim or the β-adrenoceptor agonist isoprenaline can lead to the conducted vasodilatation (Garland *et al.*, 2011; Takano *et al.*, 2004). Since there was an evidence that unlike $NO^{\bullet}$, NO^{-} can robustly hyperpolarize smooth muscle of rat mesenteric arteries (Favaloro *et al.*, 2009), we decided to test the hypothesis that NO^{-} can also produce a conducted response. In PE- preconstricted arteries, perfusion of the HNO donor Angeli's salt (30 μM) via Branch 1 evoked conducted dilatation along the Feed branch (Figure 7.9A), which had a similar magnitude as the response produced by the endothelium-dependent agonist ACh (1 μM; n = 5 and n = 6, respectively; Figure 7.10A). The K^{+} channels blockers TEA (1 mM; n = 4) and 4-AP (150 μM; n = 4) suppressed the local response, proportionally reducing the conducted response. Combination of both (n = 5) or application of KCl (45 mM) as a contractile agent (n = 3) blocked local and conducted dilatation (7.9B). Whilst scavenging of HNO with L-cysteine resulted in suppression of both local and conducted responses (n = 1), endothelial denudation abolished conducted, but not local dilatation (n = 5; Figure 7.9C).

7.3.7 Endogenous NO^{-} induces local and conducted dilatation in the triple-cannulated pressurized mesenteric arteries of rat

It was recently demonstrated that eNOS can produce NO^{-} in rat mesenteric arteries (Andrews *et al.*, 2009). Therefore, we decided to evaluate whether endogenous NO^{-} can produce local dilatation that is able to evoke a similar level of conducted dilatation as the HNO donor Angeli's salt.

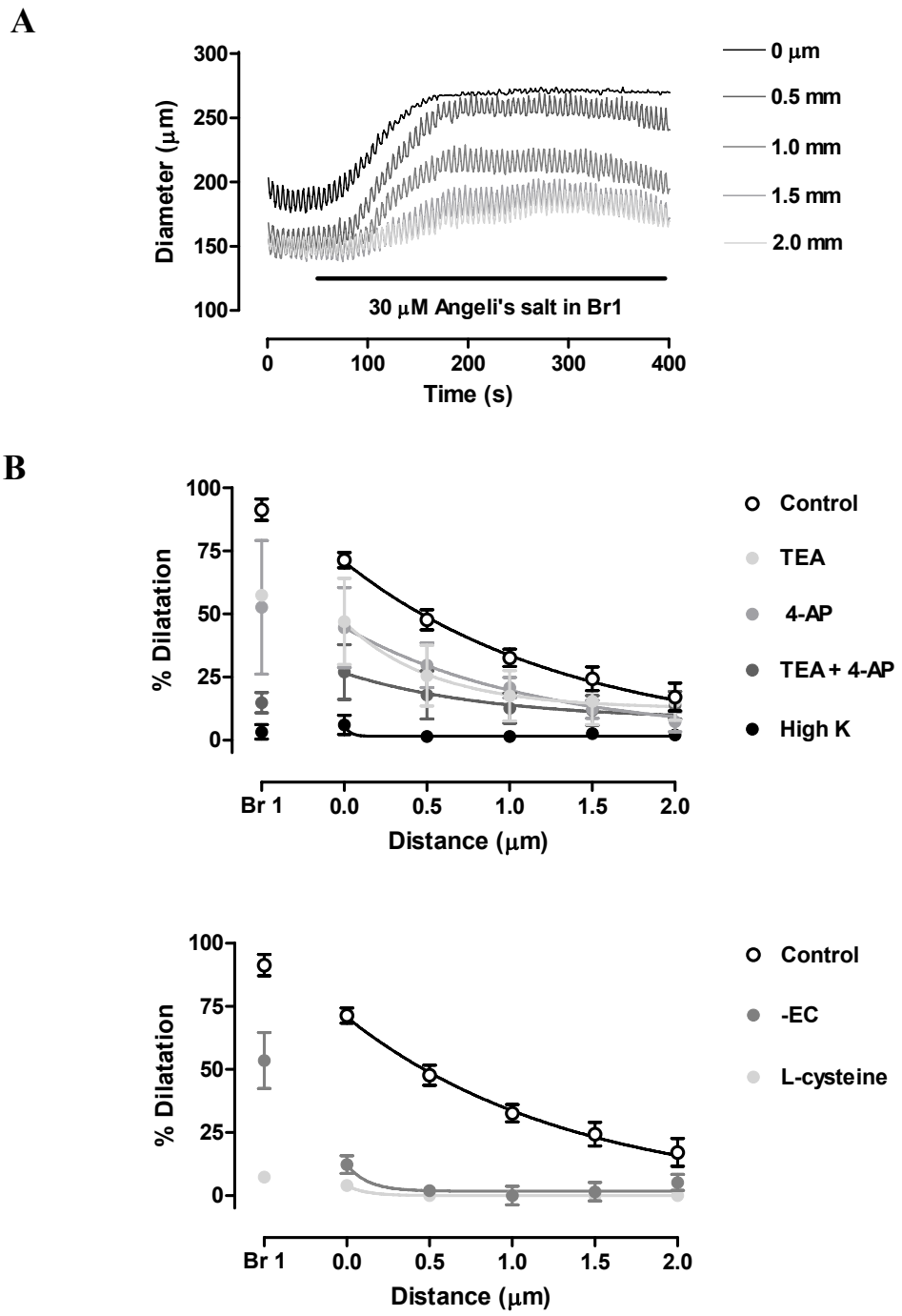


Figure 7.9 Conducted response to HNO donor Angeli's salt in the triple-cannulated small mesenteric arteries preconstricted with PE

A. Representative trace illustrating conducted dilatation to the HNO donor Angeli's salt (30 μM), perfused via Branch 1.
B. Averaged data (mean ± s.e.mean) showing effect of the K^+ channels inhibition with TEA (1 mM, n = 4), 4-AP (150 μM, n = 4) and high K^+ (45 mM, n = 3) on the conducted response to Angeli's salt.
C. Averaged data demonstrating the effect of endothelial denudation (-EC, n = 5) or NO^- scavenger L-cysteine (3 mM, n = 1) on the conducted response to Angeli's salt.

In order to unmask the eNOS-dependent pathway, EDH was blocked by SK_{Ca} and IK_{Ca} channels inhibitors apamin (50 nM) and TRAM-34 (1 μM), respectively, and a higher concentration of ACh (10 μM) was perfused via Branch 1. In PE- preconstricted arteries, ACh could still evoke a robust dilatation of the Branch 1, which was accompanied by a similar degree of spread along the Feed branch, as in control conditions (n = 4; $p>0.05$). $NO^{\bullet}$ scavenger carboxy-PTIO (200 μM) reduced local dilatation, but had hardly any effect on the conducted dilatation (n = 5). Unlike carboxy-PTIO, NO^{-} scavenger L-cysteine not only reduced local, but also abolished conducted dilatation (n = 4). Co-application of both scavengers (n = 3, data not shown) or inhibition of NOS with L-NAME (100 μM; n = 4) blocked all the responses to ACh (Figure7.10B), indicating that eNOS is likely to be the source of NO^{-} in this tissue.

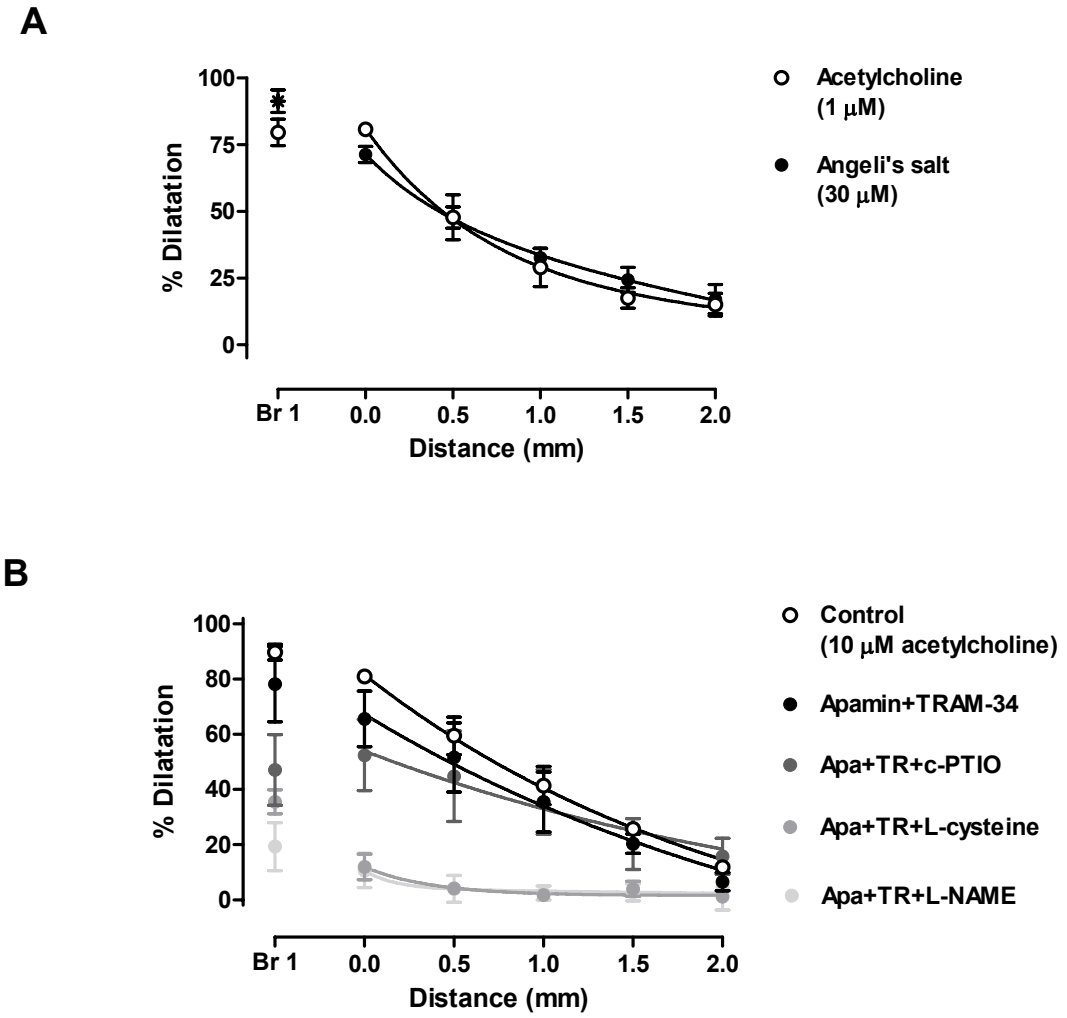


Figure 7.10 Conducted dilatation to endogenous NO^- in the small mesenteric arteries preconstricted with PE

A. Averaged data demonstrating comparable conducted responses to the HNO donor Angeli's salt (30 μM; $n = 6$) and acetylcholine (1 μM; $n = 5$).
B. Summarized data showing the effect of the $NO^•$ scavenger carboxy-PTIO (c-PTIO, 200 nM; $n = 5$), NO^- scavenger L-cysteine (3 mM), NOS inhibitor L-NAME (100 μM) on the local and conducted dilatation to acetylcholine (10 μM), remained after the EDHF pathway was inhibited with the SK_{Ca} channel inhibitor apamin (apa, 50 nM) and IK_{Ca} channel inhibitor TRAM-34 (TR; 1 μM).

Results are the mean ± s.e.mean.

7.4 Discussion

The endothelium-dependent dilatation involves formation of NO in several redox forms, among which uncharged free radical state of the NO molecule and nitroxyl anion are present (Irvine *et al.*, 2008). This study elucidates the contribution of K^+ channels in NO^- -mediated local and conducted responses in rat pressurized small mesenteric arteries.

The nitroxyl donor, Angeli's salt, evoked complete vasodilatation of pressurized arteries, with a lower pEC_{50} value than was reported in isometric conditions (in isobaric experiments pEC_{50}~5.1, whilst in wire myography pEC_{50} was between 7.0 and 7.7 (Favaloro *et al.*, 2009; Irvine *et al.*, 2003a)). Its concentration-response curve, however, exhibited lack of difference to the obtained in response to $NO^{\bullet}$, which reveal comparable effects of both NO forms on the tone of pressurized resistance arteries. Despite evidence that NO^- can be further transformed to $NO^{\bullet}$ by tissue superoxide dismutases (Fukuto *et al.*, 1993; Hobbs *et al.*, 1994; Schmidt *et al.*, 1996), the free radical scavenger carboxy-PTIO did not alter the relaxation to Angeli's salt, whilst addition of the NO^- scavenger L-cysteine markedly inhibited this response in our experiments as well as in previously published results (Ellis *et al.*, 2000; Favaloro *et al.*, 2009; Wanstall *et al.*, 2001).

From resting conditions $NO^{\bullet}$ was able to evoke hyperpolarization, which was sensitive to inhibition of K_{ATP} (Garland *et al.*, 1992a) and K_{Ca} channels (Mistry *et al.*, 1998; Sampson *et al.*, 2001). Although $NO^{\bullet}$ did not repolarize (Garland *et al.*, 1992a), or had a very modest repolarization of rat mesenteric arteries preconstricted with the α_1-adrenergic agonist (Favaloro *et al.*, 2009), the NO^- donor was able to repolarize PE-preconstricted arteries (Favaloro *et al.*, 2009), demonstrating a different mechanism of modulation of membrane potential than $NO^{\bullet}$.

A role for endogenous NO^- has recently been shown in rat mesenteric arteries, where it was able to hyperpolarize smooth muscle via activation of K_v channels (Andrews *et al.*, 2009). In accordance to this, in our experiments 4-AP inhibited dilatation to the NO^- donor, however, the BK_{Ca} channel inhibitors iberiotoxin or TEA attenuated Angeli's salt-mediated relaxation to a similar degree as 4-AP. Co-application of BK_{Ca} and K_v channel inhibitors had an additive effect, whilst ODQ alone fully blocked the response. Precontraction (via depolarization) with high K^+ also resulted in suppression of the dilatation. These results clearly demonstrate that NO^- is modulating vascular tone via the soluble guanylyl cyclase-mediated activation of BK_{Ca} and K_v channels (Yuill *et al.*, 2010).

Apart from releasing EDRF, modulation of tone by the endothelium also occurs through providing a low-resistance pathway for a passive spread of hyperpolarizing current both longitudinally and radially via the myo-endothelial gap junctions (Dora, 2001; Dora *et al.*, 2003b; Takano *et al.*, 2004; Yamamoto *et al.*, 2001). This spread of hyperpolarization results in coordination of vasomotion over significant distances along the artery wall. The essential role for the endothelium in this process is proved by denudation, which abolishes both synchronized oscillations of tone (Mauban *et al.*, 2004) and conducted dilatation in rat mesenteric arteries (Garland *et al.*, 2011; Takano *et al.*, 2004).

In PE- preconstricted arteries, oscillations of tone were markedly synchronized by the NO^- donor, and this was not mimicked by $NO^\bullet$ gas. Under basal conditions, vasomotion was also sensitive to NOS inhibition as well as to NO^- scavenging, possibly, reflecting a modulating effect of basally released endogenous NO (Martin *et al.*, 1986; Simonsen *et al.*, 1999) or in response to Ca^{2+} incoming from smooth muscle cells (Dora *et al.*, 2000; Kansui *et al.*, 2008). It was shown that NO^- mediates hyperpolarization and

relaxation solely via a ODQ-sensitive pathway, whilst $NO^{\bullet}$-evoked dilatation was less dependent on cGMP (Favaloro *et al.*, 2009). This may be of importance since cGMP has been demonstrated to coordinate both smooth muscle $[Ca^{2+}]_i$ oscillations and vasomotion in rat mesenteric arteries (Rahman *et al.*, 2005). The fact that some asynchronous vasomotion still occurred during the inhibition of NO-cGMP pathway may refer to the importance of cGMP for synchronization of the vasomotion via coordination of current movement between the cells (Rahman *et al.*, 2007), but not for the initiation of the smooth muscle Ca^{2+} oscillations (Mauban *et al.*, 2004; Rahman *et al.*, 2005).

A positive effect of endogenous NO on conducted dilatation in response to focal application of ACh was shown for hamster cheek pouch arterioles, where inhibition of NOS resulted in reduction of the distance of spread (Doyle *et al.*, 1997). Our experiments, however, demonstrate that L-NAME had no detectable effect on the conducted dilatation in rat mesenteric arteries (Figure 5.11B). Since stimulation of α_1-adrenoceptors (Nilsson *et al.*, 1998) or TP receptors (Fujiwara *et al.*, 1996) may moderately depolarize rat mesenteric arteries, this result might indicate that the cGMP pathway is amplifying electrical integration in depolarized conditions, whilst playing minimal role during spread of hyperpolarization in mesenteric arteries.

On the other hand, $NO^{\bullet}$, when when released from an $NO^{\bullet}$ donor, was not able to evoke conducted dilatation in both hamster and rat resistance arteries (Doyle *et al.*, 1997; Winter *et al.*, 2007). In contrast to this, an NO^{-} donor evoked conducted dilatation similar to that produced by ACh. Moreover, endogenous NO^{-} was also capable of stimulating conducted dilatation. This effect may be explained by the fact that NO^{-} can evoke sufficient hyperpolarization of smooth muscle (Favaloro *et al.*, 2009; Irvine *et al.*, 2003a; Yuill *et al.*, 2010), as other endothelium-dependent and independent stimuli that

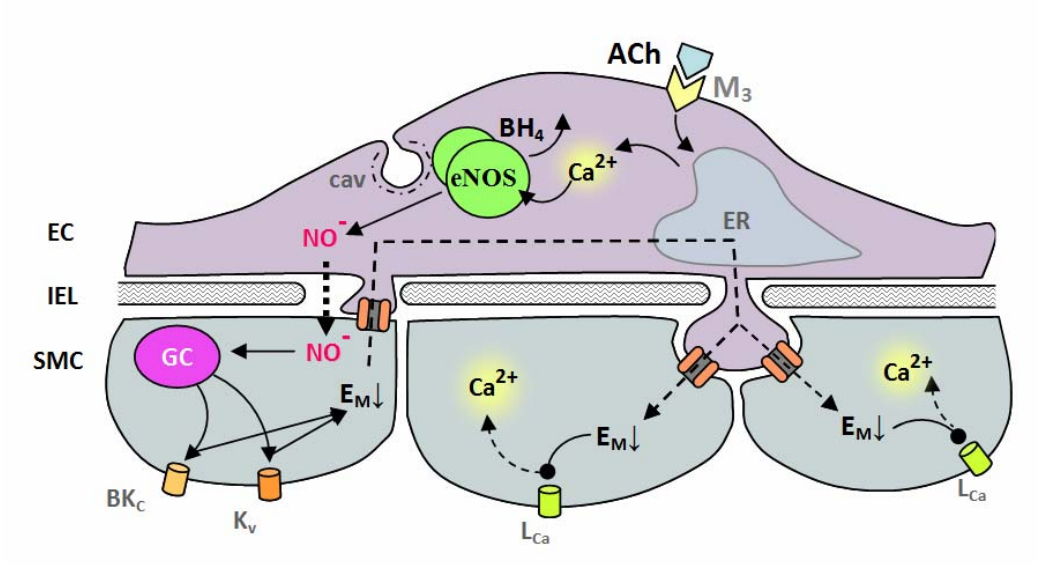


Figure 7.11 Diagram illustrating signalling of eNOS during the conducted dilatation in response to ACh

Rise in endothelial cell $[Ca^{2+}]_i$ in response to stimulation of M_3 receptor (yellow) evokes NO^- production, which hyperpolarize underlying smooth muscle cells via soluble guanylyl cyclase (magenta) signalling. This hyperpolarization can spread via gap junctions (brown) to adjacent endothelial cell and propagate to the distant smooth muscle cells, leading to their dilatation via closure of L_{Ca} channels.

led to conducted vasodilatation in this tissue (Garland *et al.*, 2011; Takano *et al.*, 2004; Winter *et al.*, 2007). The degree of conducted response was not modified by K_V and BK_{Ca} channels inhibition; however, it was minimised by endothelium denudation.

Overall, this study demonstrates that NO^- targets both K_V and BK_{Ca} channels via activation of guanylyl cyclase in pressurized resistance arteries of rat. Moreover, NO^- can be endogenously produced in sufficient amounts to modulate synchronization of vasomotion and evoke conducted vasodilatation. Despite being crucial for the local response, K^+ channels do not seem to directly participate in the conducted dilatation to NO^-, whilst the endothelium is needed to provide a pathway for the conduction of hyperpolarization produced by K_v and BK_{Ca} channels (Figure 7.11).

Chapter 8. Future directions

8.1 Exploring mechanisms for inhibition of endothelium-dependent dilatation

It is well known that β-adrenoceptors play an important role in the regulation of vascular tone (Guimaraes *et al.*, 2001). We have demonstrated that β-adrenergic signalling can not only evoke local and distant dilatation, but also interfere with the endothelial muscarinic receptor signalling, reducing involvement of eNOS and IK_{Ca} channel in the dilatation to ACh, an effect that was not mimicked by the α-adrenoceptor agonists. This process may contribute towards the development of hypertension, since this disease is associated with an increased adrenaline/NA release (Berg *et al.*, 2010; Dietz *et al.*, 1982; Klemola *et al.*, 1999; Kuklinska *et al.*, 2010). In accordance to our data, it was demonstrated that treatment with β-adrenoceptor blockers helps to attenuate arterial blood pressure via reduction of vascular resistance and increase of NO availability (Berg *et al.*, 2010; Buval'tsev *et al.*, 2003; Gupta *et al.*, 2008; Priviero *et al.*, 2007; Reiter, 2004; Wenzel *et al.*, 2009), although the receptor subtype involved is not fully determined. Generally, inhibition of both β_1 and β_2-adrenoceptors has been demonstrated to decrease blood pressure in hypertensive rats, whilst β_3-adrenoceptor inhibition was ineffective (Berg *et al.*, 2010). However, a selective β_1-adrenoceptor blocker nebivolol has been shown to lower blood pressure, improve endothelial function and NO production in human (Buval'tsev *et al.*, 2003) and rat (Wang *et al.*, 2010), whilst other β_1-adrenoceptor blockers, atenolol and metoprolol reduced blood pressure without improving endothelial function (Heffernan *et al.*, 2011). The difference between nebivolol and other β-adrenergic antagonists may lay in the ability of nebivolol to activate endothelial β_3-adrenoceptors that can stimulate eNOS (Dessy *et al.*, 2005). On the other hand, not only nebivolol, but also the selective β_2-adrenoceptor blocker ICI 118 551 reduced pulmonary vasoconstriction via activation of the NO pathway (Wenzel *et al.*, 2009). It is important to remember, though, that the effects of β-blockers

studied *in vivo* are attributed not only to the action on vascular adrenoceptors, but also on neuronal and cardiac adrenoceptors. Thereby it seems attractive to elucidate the effects of subtype-specific β-adrenergic agonists and antagonists on the inhibition of the endothelium-dependent dilatation in isolated arteries.

An important question arises regarding the localization of β-adrenoceptors. The fact that abluminal application of adrenaline resulted in a smaller shift of the concentration response curve to ACh than luminal perfusion may indicate that β-adrenoceptors responsible for the inhibition are located close to the arterial intima. There are several pieces of evidence demonstrating that of β-adrenoceptors can be expressed by endothelial cells (Broeders *et al.*, 2000; Daly *et al.*, 2010), although some of these results can be argued due to the poor selectivity of commercially available antibodies (Pradidarcheep *et al.*, 2009). A perspective approach of investigation of the receptor localization may give an adrenergic ligand labelled with fluorescent dye, such as BODIPY TMR-CGP 12177 (Briones *et al.*, 2005),

Another direction of future studies may lay in the area of establishing of the downstream signalling molecules that participate in the inhibition of the dilatation to ACh by β-adrenoceptor stimulation. It is clear that adenylyl cyclase is involved in the process since forskolin mimicked the effect of the β-adrenergic agonists, however, cAMP can activate PKA and Epac, both of which were shown to be capable to stimulate PKC (Duquesnes *et al.*, 2010; Wooten *et al.*, 1996), therefore selective PKA and Epac antagonist may be employed to examine this pathway.

We have also shown that the β-adrenergic inhibition of the ACh-mediated dilatation partly involves activation of COX. On the other hand, contribution of this enzyme in responses to isoprenaline was augmented in old and hypertensive rats. This may lead to an enhanced β-adrenergic inhibition of the endothelium-dependent

dilatation. In agreement to this hypothesis, our preliminary experiments performed in arteries from 6 months old rats revealed a greater isoprenaline-mediated shift of the ACh CRC than in 12 week old animals (from pEC_{50} = 7.23 ± 0.05 to pEC_{50} = 6.23 ± 0.05, *n* = 4; Figure 8.1), although arteries from SHRs were not tested yet. Evidence that reduced blood plasma NO levels in SHRs is restored by β-adrenoceptor blockers (Wang *et al.*, 2010), whilst COX signalling in this strain is usually increased (Feletou *et al.*, 2010b), makes it feasible that β-adrenergic inhibition of the endothelium-dependent dilatation may be also augmented in hypertensive rats due to enhanced prostanoid formation.

All prostanoids are able to activate TP receptor (Feletou *et al.*, 2010a), and the signalling pathway involves PKC (Perez-Vizcaino *et al.*, 1997; Zhang *et al.*, 2010). Both TP receptor (Feletou *et al.*, 2010b) and PKC (Johnsen *et al.*, 2005) are implicated in cardiovascular pathogenesis; however, whether this receptor is responsible for the β-adrenoceptor-mediated inhibition of the dilatation to ACh and which prostanoid may be involved is not yet known. It is also of interest to elucidate if other agonists that activate PKC exhibit similar inhibitory action on the dilatation to ACh, as β-adrenergic agonists do. Endothelin-1 and angiotensin II were shown to activate PKC in rat mesenteric arteries (Rainbow *et al.*, 2009). So far we have tested endothelin-1, -3, and angiotensin II, and discovered a significant shift of the ACh CRC when the agonists were luminally perfused (Figure 8.2). Interestingly, whilst endothelin-3 had a similar effect regardless luminal or abluminal application, endothelin-1 exhibited a greater inhibition of the dilatation when applied in bath, indicating impairment of the smooth muscle reactivity rather than endothelial dysfunction in response to endothelin-1. Whether the inhibition of the dilatation to ACh by endothelin-1, endothelin-3, and angiotensin II employ similar pathway as the β-adrenergic agonists, may be revealed in ongoing studies.

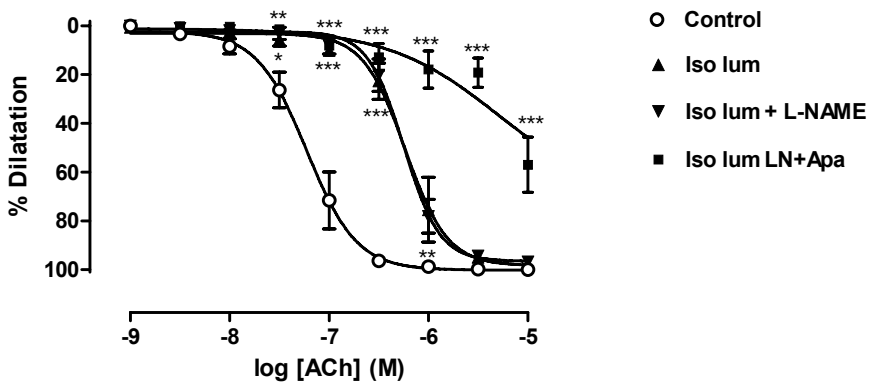


Figure 8.1 Effect of luminal isoprenaline perfusion on the dilatation to ACh in pressurized small mesenteric arteries from 6 months old rats

Averaged data demonstrating an effect of luminal perfusion of isoprenaline (1 μM; $n = 4$) followed by NOS suppression with L-NAME (100 μM; $n = 3$), and SK_{Ca} channel inhibition by apamin (50 nM; $n = 3$) on the dilatation to ACh. Whilst L-NAME had no effect, apamin suppressed the remaining dilatation.

Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01, ***p<0.001 vs. control.

Anoher endothelium-dependent dilatation pathway, which may be affected by the augmented PKC signalling, is the dilatation mediated by purinergic receptor activation. We have recently demonstrated that PKC is involved in the desensitization of the endothelial $P2Y_1$ receptor, resulting in a decreased amplitude and duration of the dilatation (Figure 8.3) (Rodriguez-Rodriguez *et al.*, 2009). Thereby it seems relevant to study if β-adrenergic signalling can amplify the desensitization of endothelial purinergic receptors.

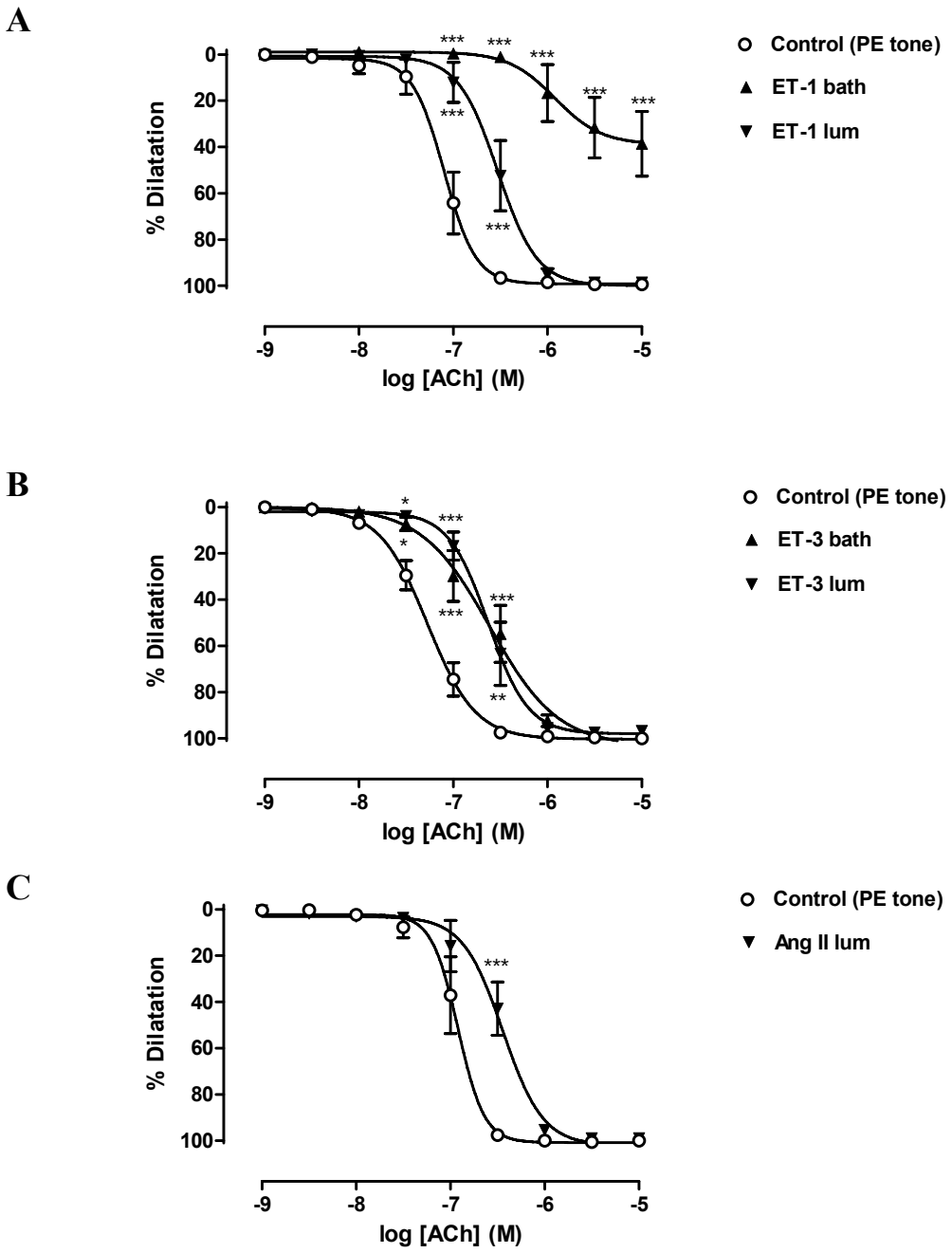


Figure 8.2 Effects of endothelin-1, endothelin-3 and angiotensin II on the dilatation to ACh of pressurized small mesenteric arteries of rat

A. Summarized concentration-response curves representing effects of luminal (lum; 10 nM) or abluminal (bath; 1 nM - 10 nM) application of endothelin-1 (ET-1) on the dilatation to ACh (1 nM-10 μM).
B. Summarized data showing effects of luminal (100 nM) or abluminal (30 nM - 300 nM) application of endothelin-3 (ET-3).
C. Summarized data showing effects of luminal application of angiotensin II (Ang II; 10 nM).
Results shown are the mean ± s.e.mean; *p<0.05, **p<0.01, ***p<0.001 vs. control.

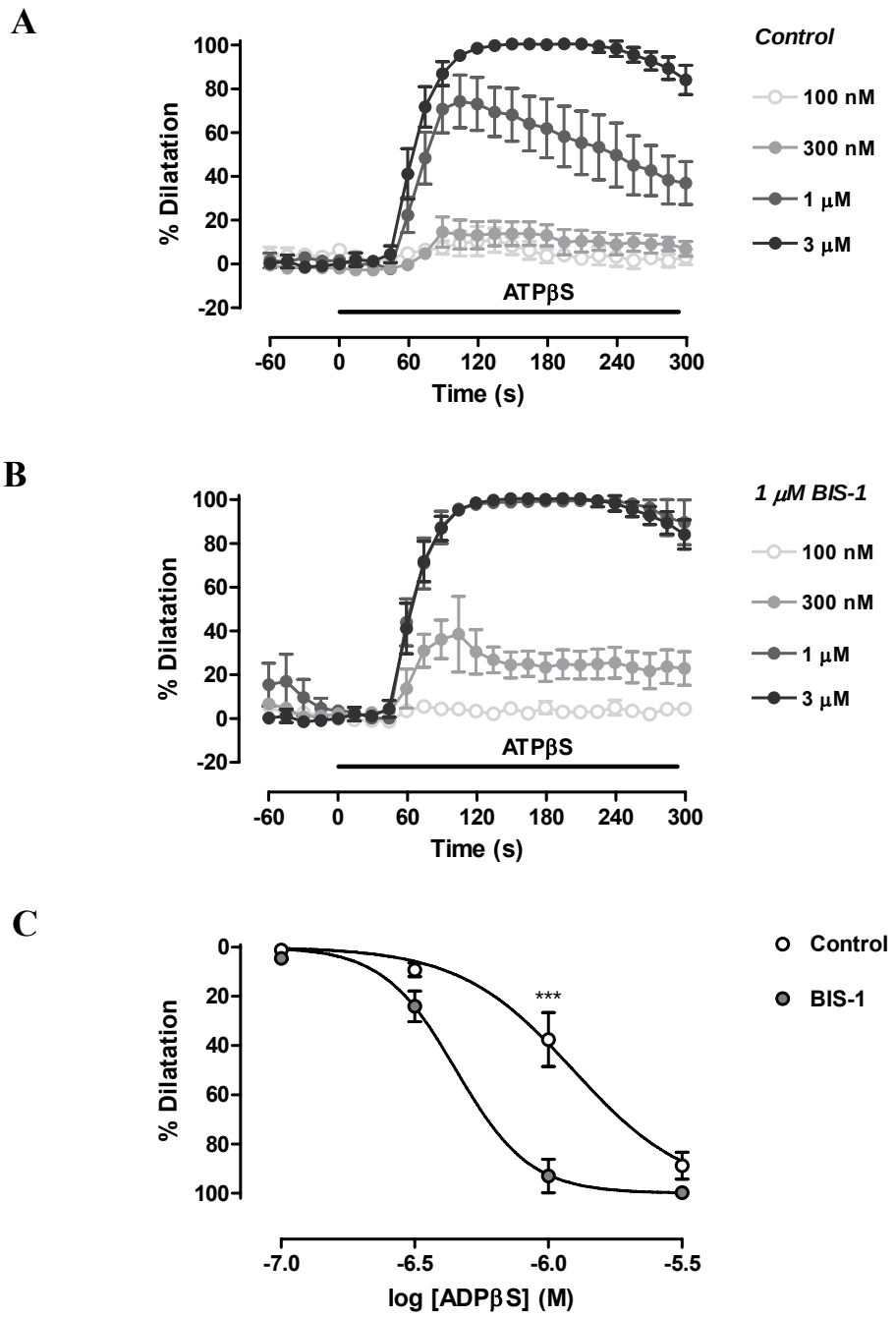


Figure 8.3 Effect of PKC inhibition on the local dilatation to ADPβS in pressurized rat mesenteric arteries

A. Averaged traces illustrating time course of the concentration-dependent dilatation to the luminal perfusion of ADPβS (100 nM-3 μM) in control.
B. Averaged traces demonstrating effect of PKC inhibitor BIS-1 (1 μM) on the concentration-dependent dilatation to luminal perfusion of ADPβS.
C. Summarized data of the effect of BIS-1 on the dilatation to ADPβS taken at five minutes after the start of luminal perfusion of ADPβS.
Results shown are the mean ± s.e.mean; ***p<0.001 vs. control.
(Data modified from Rodriguez-Rodriguez *et al.*, 2009).

8.2 Exploring pathways of the β-adrenergic-mediated COX activation

Our experiments demonstrated a prominent indomethacin-sensitive component of the response to β-adrenergic agonists that led to suppression of the dilatation in old and hypertensive rats. Despite the dilatation of the mesenteric arteries from young SHRs did not exhibit a significant difference in comparison to control normotensive rats (Blankesteijn *et al.*, 1996), membrane potential measurements have shown an impaired hyperpolarization to isoprenaline in mesenteric arteries from not only old (Fujii et al., 1999), but also young SHRs (Goto et al., 2001), demonstrating that β-adrenergic hyperpolarization may be defective before the development of altered functional response. Therefore it seems worth clarifying whether COX participates in the reduction of the hyperpolarization in both age and disease groups.

The mechanisms of how β-adrenergic signalling can lead to stimulation of COX are not yet clarified. Whilst COX is unlikely to be directly phosphorylated by PKC or PKA (Vezza *et al.*, 1996), enhanced signalling may result from augmentation of COX expression (Choudhary *et al.*, 2004; Steinert *et al.*, 2009) as well as in response to $[Ca^{2+}]_i$ rise (Tang *et al.*, 2007). We have preliminary data showing that application of isoprenaline evoked increase in number of endothelial cells that displayed Ca^{2+} transients (in control, 25.3±3.8%, n = 4, whilst after 10 minutes of isoprenaline pre-incubation 47.1 ± 7.2%, n = 4, cells had Ca^{2+} transients; Figure 7.4A). Whether this transients were the consequence of endothelial or smooth muscle β-adrenoceptor signalling is not clear yet, however, inhibition of phospholipase C by U73122 (1 μM) suppressed the response (n = 1), suggesting the involvement of Epac (Schmidt *et al.*, 2001). Intriguingly, Ca^{2+} transients in response to isoprenaline were also suppressed by the NCX inhibitor benzamil (50 μM; 4.7 ± 4.7% cells had Ca^{2+} transients, n = 3). This

observation may be explained by the observation that NCX can be up-regulated by both PKA (Zhang *et al.*, 2009b) and PKC (Harper *et al.*, 2010), enhancing Ca^{2+} influx.

It is worth considering also that COX exerts its actions via metabolising arachidonic acid, which is produced by phospholipase A. Thereby, we may speculate the potential involvement of the arachidonic acid pathway, which is supported by several facts. First, it was shown that stimulation of β-adrenoceptors leads to release of arachidonic acid in rat hearts (Neuman *et al.*, 2002) as well as in human monocytes (Borda *et al.*, 1998); second, PLA can be activated by PKC (Xing *et al.*, 1992), a possible downstream enzyme of adenylyl cyclase signalling (Duquesnes *et al.*, 2010; Wooten *et al.*, 1996); third, PLA was shown to be overactivated (Kato *et al.*, 1992) and participate in the EDCF release in SHRs (Wong *et al.*, 2010). Therefore, it may be possible that raised arachidonic acid levels in response to G_s-protein coupled receptor stimulation are involved in the release of COX metabolites and development of hypertension.

Apart from COX, arachidonic acid can be metabolized by other enzymes, such as cytochrome P450 epoxygenases, which results in generation of EETs. EETs can exert diverse actions on vascular function, among which is the action as an endogenous antagonist for TP receptor in rat arteries (Behm *et al.*, 2009) as well as inhibition of COX-2 in rat monocytes (Kozak *et al.*, 2003). On the other hand, a recent publication has highlighted a beneficial effect of cytochrome P450 epoxygenases expression on the prevention of hypertension development in hypertensive rat model (Xiao *et al.*, 2010). All these lines of evidence may help to explain not only the effect of EETs in the reduction of inflammation (Behm *et al.*, 2009), but also contribute to the evaluation of pathways that lead to altered COX signalling and may underlay endothelial dysfunction.

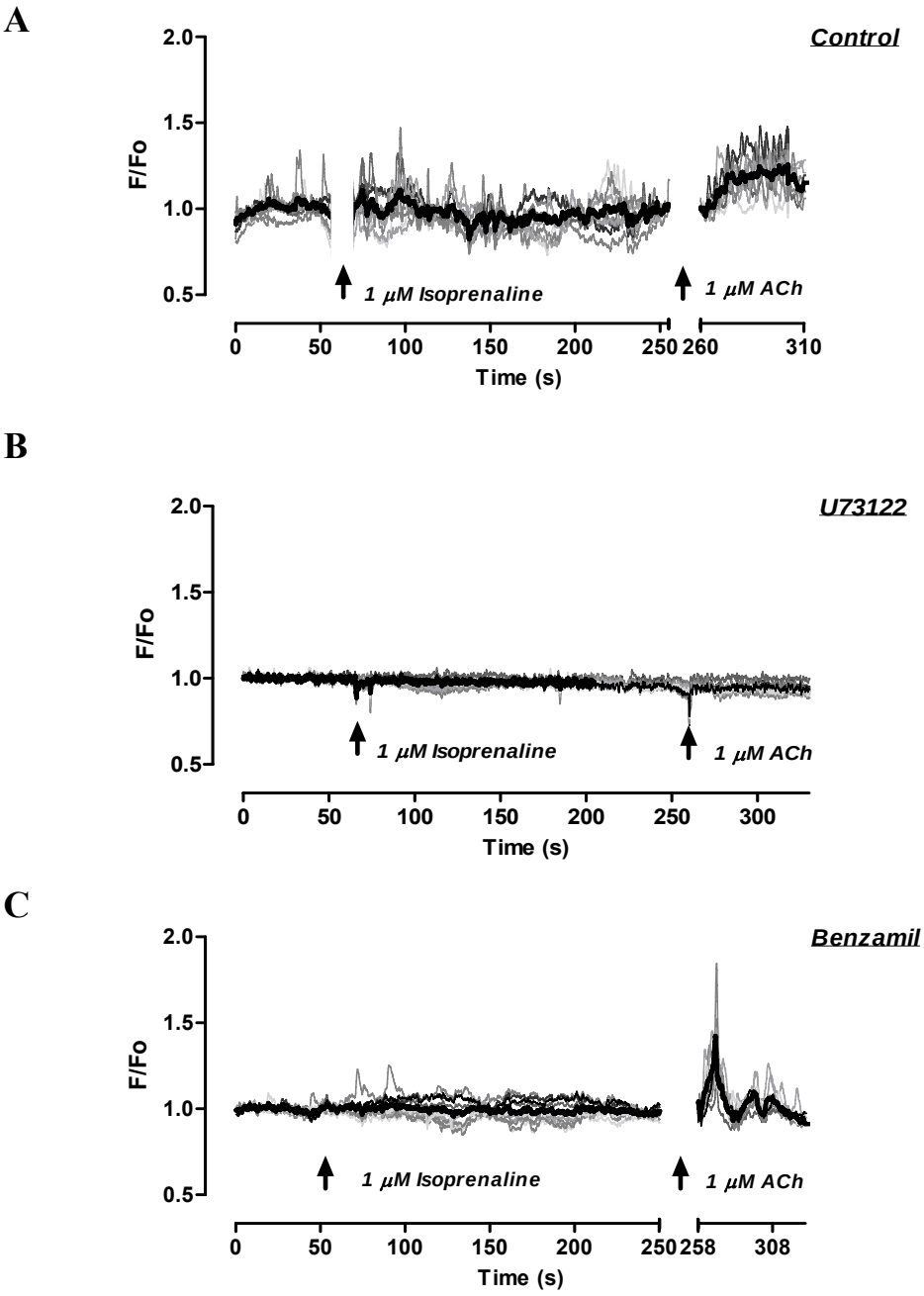


Figure 8.4 Effects of PLC and NCX inhibition on the isoprenaline -mediated rise in the EC $[Ca^{2+}]_i$ of pressurized small mesenteric arteries of rat

A. Representative trace of fluorescence intensity changes (F/F_0) of endothelial cells loaded with Oregon Green 488 BAPTA-1 AM in response to isoprenaline (1 μM) and ACh (1 μM). Responses of individual cells are shown with grey lines; average intensity is represented by a thick black line.
B. Effect of the PLC inhibitor U73122 (1 μM) on the $[Ca^{2+}]_i$ rise in response to isoprenaline and ACh.
C. Effect of the NCX inhibitor benzamil (50 μM) on the $[Ca^{2+}]_i$ changes in response to isoprenaline and ACh.

Chapter 9. Conclusions

The study presented within this thesis aimed to evaluate the role of the endothelium in local and distant regulation of vascular tone following activation of endothelium-dependent and -independent signalling pathways.

Firstly, we have examined whether the endothelium participates in the dilatation to physiological adrenergic agonists, adrenaline and NA. It has been suggested for several arterial beds that β-adrenergic signalling can lead to NO production and thereby the endothelium may directly facilitate the resulting dilatory response (Ferro *et al.*, 2004; Figueroa *et al.*, 2009b). Our results, however, demonstrate that the endothelium does not directly participate in the local dilatation to β-adrenoceptor agonists by release of NO or EDHF. Moreover, the endothelium was implicated in the reduction of the dilatation, seemingly, by activation of COX. Stimulation of TP receptors also suppressed β-adrenoceptor signalling, suggesting that COX products may act on TP receptors. Experiments performed on 6 months old and hypertensive animals revealed exaggerated COX-mediated inhibition of the dilatation to β-adrenoceptor agonists, providing an insight to the possible mechanisms of the vascular dysfunction development observed during aging and hypertension.

It was demonstrated also that the endothelium restricted luminally applied adrenergic agonists from acting on vascular β-adrenoceptors, indicating that endothelial cells are the first to come into contact with adrenergic agonists carried by blood in vivo. The fact that endothelial cells do express adrenoceptors (Briones *et al.*, 2005) may provide an additional regulatory mechanism of endothelial cell signalling. One of the key findings of this study reveals the pathway of NA/adrenaline-mediated reduction of the local dilatation to ACh. The main role seems to belong to β-adrenoceptors, which through activation of adenylyl cyclase eliminate eNOS- and IK_{Ca} channel-dependent

components of the dilatation. Additionally, a role for PKC and COX was suggested. This result may be extrapolated to certain cardiovascular diseases associated with high plasma NA/adrenaline levels and reduced endothelium-dependent dilatation.

Activation of endothelial muscarinic receptors by local application of ACh evoked prominent conducted dilatation. This phenomenon depended on the integrity of endothelium as well as functional K^+ channels that were activated during EDHF or NO^- signalling, indicating that the predominant vasodilator stimuli that propagated along the vessel wall, was the hyperpolarization. Additionally, despite attenuating the local dilatation, TP and β-adrenergic receptor agonists enhanced the conducted dilatation.

Another major finding of the present work was the discovery of the ability of endothelium-independent agonists, adrenaline and NA, to evoke conducted dilatation that spreads along the vessel wall similar to the dilatation in response to endothelium-dependent agonist ACh. The main role belonged to classical β-adrenoceptors, whereas activation of α-adrenoceptors alone did not cause the conducted response. This result provides a physiological relevance to the described previously ability of levcromakalim to evoke conducted dilatation (Takano *et al.*, 2004): although K_{ATP} channels did not contribute significantly to the local response, they were essential for the spreading dilatation. Importantly, whilst the endothelium served to reduce the β-adrenergic dilatation, it was crucial for the propagated response.

Lastly, it was herein demonstrated that apart from $NO^{\bullet}$, the endothelium can also endogenously produce NO^- in amounts sufficient to modulate synchronization of vasomotion and evoke conducted dilatation. This dilatation was enabled by guanylyl cyclase-mediated opening of K_V and BK_{Ca} channels, and, again, the endothelium was needed to provide a pathway for the hyperpolarization produced by K^+ channels.

Overall, the results described in this thesis reveal the role of the endothelium in regulation of not only local arterial tone, by release of EDCF, EDHF, NO^-, and $NO^\bullet$, but also tone of remote smooth muscle cells, via providing a pathway for a conducted vasodilatory signal (presumably, hyperpolarization). Dysfunction of the endothelium, which may develop in response to raised plasma catecholamine levels, may help to understand the precursors leading to development of hypertension.

Chapter 10. References

ADAK, S., WANG, Q. & STUEHR, D.J. (2000). Arginine conversion to nitroxide by tetrahydrobiopterin-free neuronal nitric-oxide synthase. Implications for mechanism. *J Biol Chem*, **275,** 33554-61.

ADEAGBO, A.S. & TRIGGLE, C.R. (1993). Varying extracellular [K+]: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. *J Cardiovasc Pharmacol*, **21,** 423-9.

AHLQUIST, R.P. (1948). A study of the adrenotropic receptors. *Am J Physiol*, **153,** 586-600.

AIELLO, E.A., MALCOLM, A.T., WALSH, M.P. & COLE, W.C. (1998). Beta-adrenoceptor activation and PKA regulate delayed rectifier K+ channels of vascular smooth muscle cells. *Am J Physiol*, **275,** H448-59.

AKATA, T., KANNA, T., YOSHINO, J. & TAKAHASHI, S. (2003). Mechanisms of direct inhibitory action of isoflurane on vascular smooth muscle of mesenteric resistance arteries. *Anesthesiology*, **99,** 666-77.

AKIMOTO, Y., HORINOUCHI, T., SHIBANO, M., MATSUSHITA, M., YAMASHITA, Y., OKAMOTO, T., YAMAKI, F., TANAKA, Y. & KOIKE, K. (2002). Nitric oxide (NO) primarily accounts for endothelium-dependent component of beta-adrenoceptor-activated smooth muscle relaxation of mouse aorta in response to isoprenaline. *J Smooth Muscle Res*, **38,** 87-99.

ALBERT, A.P., SALEH, S.N. & LARGE, W.A. (2008). Inhibition of native TRPC6 channel activity by phosphatidylinositol 4,5-bisphosphate in mesenteric artery myocytes. *J Physiol*, **586,** 3087-95.

ALEY, P.K., PORTER, K.E., BOYLE, J.P., KEMP, P.J. & PEERS, C. (2005). Hypoxic modulation of Ca2+ signaling in human venous endothelial cells. Multiple roles for reactive oxygen species. *J Biol Chem*, **280,** 13349-54.

AMATORE, C., ARBAULT, S., DUCROCQ, C., HU, S. & TAPSOBA, I. (2007). Angeli's salt (Na2N2O3) is a precursor of HNO and NO: a voltammetric study of the reactive intermediates released by Angeli's salt decomposition. *ChemMedChem*, **2,** 898-903.

ANDREWS, K.L., IRVINE, J.C., TARE, M., APOSTOLOPOULOS, J., FAVALORO, J.L., TRIGGLE, C.R. & KEMP-HARPER, B.K. (2009). A role for nitroxyl (HNO) as an endothelium-derived relaxing and hyperpolarizing factor in resistance arteries. *Br J Pharmacol*, **157,** 540-50.

ANDRIANTSITOHAINA, R., LAGAUD, G.J., ANDRE, A., MULLER, B. & STOCLET, J.C. (1995). Effects of cGMP on calcium handling in ATP-stimulated rat resistance arteries. *Am J Physiol*, **268,** H1223-31.

ARAS-LOPEZ, R., XAVIER, F.E., FERRER, M. & BALFAGON, G. (2009). Dexamethasone decreases neuronal nitric oxide release in mesenteric arteries from hypertensive rats through decreased protein kinase C activation. *Clin Sci (Lond)*, **117,** 305-12.

ARCHER, S.L., HUANG, J.M., HAMPL, V., NELSON, D.P., SHULTZ, P.J. & WEIR, E.K. (1994). Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci U S A*, **91,** 7583-7.

ARSHAD, M., VIJAY, V., FLOYD, B.C., MARKS, B., SARABU, M.R., WOLIN, M.S. & GUPTE, S.A. (2006). Thromboxane receptor stimulation suppresses guanylate cyclase-mediated relaxation of radial arteries. *Ann Thorac Surg*, **81,** 2147-54.

ARVOLA, P., PORSTI, I., VUORINEN, P., HUHTALA, H., METSA-KETELA, T. & VAPAATALO, H. (1992). L-cysteine augments the vasorelaxation induced by sodium nitrite and SIN-1 but not that due to acetylcholine. *Eur J Pharmacol*, **214,** 289-92.

BACHETTI, T., COMINI, L., AGNOLETTI, L., PEDERSINI, P., GAIA, G., CARGNONI, A., BELLET, M., CURELLO, S. & FERRARI, R. (1998). Effects of chronic noradrenaline on the nitric oxide pathway in human endothelial cells. *Basic Res Cardiol*, **93,** 250-6.

BALL, C.J., WILSON, D.P., TURNER, S.P., SAINT, D.A. & BELTRAME, J.F. (2009). Heterogeneity of L- and T-channels in the vasculature: rationale for the efficacy of combined L- and T-blockade. *Hypertension*, **53,** 654-60.

BAO, X., LEE, S.C., REUSS, L. & ALTENBERG, G.A. (2007). Change in permeant size selectivity by phosphorylation of connexin 43 gap-junctional hemichannels by PKC. *Proc Natl Acad Sci U S A*, **104,** 4919-24.

BARBIERI, A., ZONTA, F., SARACINO, M.L., DONDI, G., FRATTINI, P., LUCCHELLI, A., SANTAGOSTINO, M., D'AGOSTINO, G., MAESTRI, M.O. & ZONTA, A. (1996). Evaluation of the reperfusion syndrome after liver ischemia in the rat. *J Surg Res*, **62,** 153-8.

BARTBERGER, M.D., LIU, W., FORD, E., MIRANDA, K.M., SWITZER, C., FUKUTO, J.M., FARMER, P.J., WINK, D.A. & HOUK, K.N. (2002). The reduction potential of nitric oxide (NO) and its importance to NO biochemistry. *Proc Natl Acad Sci U S A*, **99,** 10958-63.

BARYSHNIKOV, S.G., PULINA, M.V., ZULIAN, A., LINDE, C.I. & GOLOVINA, V.A. (2009). Orai1, a critical component of store-operated Ca2+ entry, is functionally associated with Na+/Ca2+ exchanger and plasma membrane Ca2+ pump in proliferating human arterial myocytes. *Am J Physiol Cell Physiol*, **297,** C1103-12.

BEECH, D.J., MURAKI, K. & FLEMMING, R. (2004). Non-selective cationic channels of smooth muscle and the mammalian homologues of Drosophila TRP. *J Physiol*, **559,** 685-706.

BEGONHA, R., MOURA, D. & GUIMARAES, S. (1995). Vascular beta-adrenoceptor-mediated relaxation and the tone of the tissue in canine arteries. *J Pharm Pharmacol*, **47,** 510-3.

BELEZNAI, T., Z. , YAROVA, P.L., YUILL, K.H. & DORA, K.A. (2011). Smooth muscle Ca2+-activated and voltage-gated K+ channels modulate conducted dilation in rat isolated small mesenteric arteries. *Microcirculation*.

BELHASSEN, L., PELLE, G., DUBOIS-RANDE, J.L. & ADNOT, S. (2003). Improved endothelial function by the thromboxane A2 receptor antagonist S 18886 in patients with coronary artery disease treated with aspirin. *J Am Coll Cardiol*, **41,** 1198-204.

BEN CHEIKH, R., FERAY, J.C., ALAOUI, A. & CROZATIER, B. (2002). Thromboxane A2 in vasomotor effects of phenylephrine, acetylcholine, and bradykinin in rat mesenteric bed. *J Cardiovasc Pharmacol*, **40,** 255-64.

BENTHEM, L., VAN DER LEEST, J., STEFFENS, A.B. & ZIJLSTRA, W.G. (1995). Metabolic and hormonal responses to adrenoceptor antagonists in exercising rats. *Metabolism*, **44,** 245-53.

BENY, J.L. & VON DER WEID, P.Y. (1991). Hydrogen peroxide: an endogenous smooth muscle cell hyperpolarizing factor. *Biochem Biophys Res Commun*, **176,** 378-84.

BERG, T., PIERCEY, B.W. & JENSEN, J. (2010). Role of beta1-3-adrenoceptors in blood pressure control at rest and during tyramine-induced norepinephrine release in spontaneously hypertensive rats. *Hypertension*, **55,** 1224-30.

BERNATOWA, I., CSIZMADIOVA, Z., KOPINCOVA, J. & PUZSEROVA, A. (2007). Vascular function and nitric oxide production in chronic social-stress-exposed rats with various family history of hypertension. *J Physiol Pharmacol*, **58,** 487-501.

BERRA-ROMANI, R., MAZZOCCO-SPEZZIA, A., PULINA, M.V. & GOLOVINA, V.A. (2008). Ca2+ handling is altered when arterial myocytes progress from a contractile to a proliferative phenotype in culture. *Am J Physiol Cell Physiol*, **295,** C779-90.

BERRA-ROMANI, R., RAQEEB, A., GUZMAN-SILVA, A., TORRES-JACOME, J., TANZI, F. & MOCCIA, F. (2010). Na+-Ca2+ exchanger contributes to Ca2+ extrusion in ATP-stimulated endothelium of intact rat aorta. *Biochem Biophys Res Commun*, **395,** 126-30.

BERRIDGE, M.J. (1995). Capacitative calcium entry. *Biochem J*, **312 (Pt 1),** 1-11.

BERRIDGE, M.J. & IRVINE, R.F. (1989). Inositol phosphates and cell signalling. *Nature*, **341,** 197-205.

BERRIDGE, T.L. & ROACH, A.G. (1986). Characterization of alpha-adrenoceptors in the vasculature of the canine nasal mucosa. *Br J Pharmacol*, **88,** 345-54.

BEVAN, J.A. & OSHER, J.V. (1972). A direct method for recording tension changes in the wall of small blood vessels in vitro. *Agents Actions*, **2,** 257-60.

BEVILACQUA, M., VAGO, T., MONOPOLI, A., BALDI, G., FORLANI, A., ANTONA, C., BIGLIOLI, P., SCROFANI, R. & NORBIATO, G. (1991). Alpha 1 adrenoceptor subtype mediates noradrenaline induced contraction of the human internal mammary artery: radioligand and functional studies. *Cardiovasc Res*, **25,** 290-4.

BIRUKOVA, A.A., BURDETTE, D., MOLDOBAEVA, N., XING, J., FU, P. & BIRUKOV, K.G. (2009). Rac GTPase is a hub for protein kinase A and Epac signaling in endothelial barrier protection by cAMP. *Microvasc Res*, **79,** 128-38.

BLACK, J.W., DUNCAN, W.A. & SHANKS, R.G. (1965). Comparison of some properties of pronethalol and propranolol. *Br J Pharmacol Chemother*, **25,** 577-91.

BLANCO-RIVERO, J., ARAS-LOPEZ, R., DEL CAMPO, L., SAGREDO, A., BALFAGON, G. & FERRER, M. (2006a). Orchidectomy increases beta-adrenoceptor activation-mediated neuronal nitric oxide and noradrenaline release in rat mesenteric artery. *Neuroendocrinology*, **84,** 378-85.

BLANCO-RIVERO, J., BALFAGON, G. & FERRER, M. (2006b). Orchidectomy modulates alpha2-adrenoceptor reactivity in rat mesenteric artery through increased thromboxane A2 formation. *J Vasc Res*, **43,** 101-8.

BLANKESTEIJN, W.M. & THIEN, T. (1993). Effect of NG-monomethyl-L-arginine on the beta-adrenoceptor-mediated relaxation of rat mesenteric resistance arteries. *Life Sci*, **52,** PL135-9.

BLANQUET, P.R. (1998). Neurotrophin-induced activation of casein kinase 2 in rat hippocampal slices. *Neuroscience*, **86,** 739-49.

BOCKMAN, C.S., GONZALEZ-CABRERA, I. & ABEL, P.W. (1996). Alpha-2 adrenoceptor subtype causing nitric oxide-mediated vascular relaxation in rats. *J Pharmacol Exp Ther*, **278,** 1235-43.

BOEDTKJER, D.M., MATCHKOV, V.V., BOEDTKJER, E., NILSSON, H. & AALKJAER, C. (2008). Vasomotion has chloride-dependency in rat mesenteric small arteries. *Pflugers Arch*, **457,** 389-404.

BOITTIN, F.X., MACREZ, N., HALET, G. & MIRONNEAU, J. (1999). Norepinephrine-induced Ca(2+) waves depend on InsP(3) and ryanodine receptor activation in vascular myocytes. *Am J Physiol*, **277,** C139-51.

BOLLA, M., YOU, D., LOUFRANI, L., LEVY, B.I., LEVY-TOLEDANO, S., HABIB, A. & HENRION, D. (2004). Cyclooxygenase involvement in thromboxane-dependent contraction in rat mesenteric resistance arteries. *Hypertension*, **43,** 1264-9.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368,** 850-3.

BOLTON, T.B., LANG, R.J. & TAKEWAKI, T. (1984). Mechanisms of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. *J Physiol*, **351,** 549-72.

BONDARENKO, A. (2004). Sodium-calcium exchanger contributes to membrane hyperpolarization of intact endothelial cells from rat aorta during acetylcholine stimulation. *Br J Pharmacol*, **143,** 9-18.

BORDA, E.S., TENENBAUM, A., SALES, M.E., RUMI, L. & STERIN-BORDA, L. (1998). Role of arachidonic acid metabolites in the action of a beta adrenergic agonist on human monocyte phagocytosis. *Prostaglandins Leukot Essent Fatty Acids*, **58,** 85-90.

BORHANI, A.A., HOUSHMAND, G., SAMINI, M., NAMIRANIAN, K., HAJRASOULIHA, A.R., TAVAKOLI, S., EBRAHIMI, F. & DEHPOUR, A.R. (2005). alpha2-Adrenoceptor subsensitivity in mesenteric vascular bed of cholestatic rats: the role of nitric oxide and endogenous opioids. *Eur J Pharmacol*, **514,** 183-9.

BORISOVA, L., WRAY, S., EISNER, D.A. & BURDYGA, T. (2009). How structure, Ca signals, and cellular communications underlie function in precapillary arterioles. *Circ Res*, **105,** 803-10.

BORKOWSKI, K.R., GROS, R. & SCHNEIDER, H. (1992). Vascular beta-adrenoceptor-mediated responses in hypertension and ageing in rats. *J Auton Pharmacol*, **12,** 389-401.

BORKOWSKI, K.R. & QUINN, P. (1985). Adrenaline and the development of spontaneous hypertension in rats. *J Auton Pharmacol*, **5,** 89-100.

BOS, J.L. (2006). Epac proteins: multi-purpose cAMP targets. *Trends Biochem Sci*, **31,** 680-6.

BOS, J.L. (2003). Epac: a new cAMP target and new avenues in cAMP research. *Nat Rev Mol Cell Biol*, **4,** 733-8.

BRADLEY, A.B. & MORGAN, K.G. (1987). Alterations in cytoplasmic calcium sensitivity during porcine coronary artery contractions as detected by aequorin. *J Physiol*, **385,** 437-48.

BRAHLER, S., KAISTHA, A., SCHMIDT, V.J., WOLFLE, S.E., BUSCH, C., KAISTHA, B.P., KACIK, M., HASENAU, A.L., GRGIC, I., SI, H., BOND, C.T., ADELMAN, J.P., WULFF, H., DE WIT, C., HOYER, J. & KOHLER, R. (2009). Genetic deficit of SK3 and IK1 channels disrupts the endothelium-derived hyperpolarizing factor vasodilator pathway and causes hypertension. *Circulation*, **119,** 2323-32.

BRAHMADEVARA, N., SHAW, A.M. & MACDONALD, A. (2003). Evidence against beta 3-adrenoceptors or low affinity state of beta 1-adrenoceptors mediating relaxation in rat isolated aorta. *Br J Pharmacol*, **138,** 99-106.

BRATZ, I.N. & KANAGY, N.L. (2004). Nitric oxide synthase-inhibition hypertension is associated with altered endothelial cyclooxygenase function. *Am J Physiol Heart Circ Physiol*, **287,** H2394-401.

BRAWLEY, L., SHAW, A.M. & MACDONALD, A. (2000). Beta 1-, beta 2- and atypical beta-adrenoceptor-mediated relaxation in rat isolated aorta. *Br J Pharmacol*, **129,** 637-44.

BREDT, D.S. & SNYDER, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A*, **87,** 682-5.

BRIONES, A.M., DALY, C.J., JIMENEZ-ALTAYO, F., MARTINEZ-REVELLES, S., GONZALEZ, J.M., MCGRATH, J.C. & VILA, E. (2005). Direct demonstration of beta1- and evidence against beta2- and beta3-adrenoceptors, in smooth muscle cells of rat small mesenteric arteries. *Br J Pharmacol*, **146,** 679-91.

BROEDERS, M.A., DOEVENDANS, P.A., BEKKERS, B.C., BRONSAER, R., VAN GORSEL, E., HEEMSKERK, J.W., EGBRINK, M.G., VAN BREDA, E., RENEMAN, R.S. & VAN DER ZEE, R. (2000). Nebivolol: a third-generation beta-blocker that augments vascular nitric oxide release: endothelial beta(2)-adrenergic receptor-mediated nitric oxide production. *Circulation*, **102,** 677-84.

BROWN, M.J. & MACQUIN, I. (1981). Is adrenaline the cause of essential hypertension? *Lancet*, **2,** 1079-82.

BRUEGGEMANN, L.I., MARKUN, D.R., HENDERSON, K.K., CRIBBS, L.L. & BYRON, K.L. (2006). Pharmacological and electrophysiological characterization of store-operated currents and capacitative Ca(2+) entry in vascular smooth muscle cells. *J Pharmacol Exp Ther*, **317,** 488-99.

BUCKNER, S.A., OHEIM, K.W., MORSE, P.A., KNEPPER, S.M. & HANCOCK, A.A. (1996). Alpha 1-adrenoceptor-induced contractility in rat aorta is mediated by the alpha 1D subtype. *Eur J Pharmacol*, **297,** 241-8.

BUDZYN, K., PAULL, M., MARLEY, P.D. & SOBEY, C.G. (2006). Segmental differences in the roles of rho-kinase and protein kinase C in mediating vasoconstriction. *J Pharmacol Exp Ther*, **317,** 791-6.

BUVAL'TSEV, V.I., SPASSKAIA, M.B., NEBIERIDZE, D.V., METEL'SKAIA, V.A. & GUSEVA, O.I. (2003). [Pharmacological modulation of NO synthesis in patients with arterial hypertension and endothelial dysfunction]. *Klin Med (Mosk)*, **81,** 51-5.

CAMPBELL, W.B. & FLEMING, I. (2010). Epoxyeicosatrienoic acids and endothelium-dependent responses. *Pflugers Arch*, **459,** 881-95.

CAMPBELL, W.B., GEBREMEDHIN, D., PRATT, P.F. & HARDER, D.R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res*, **78,** 415-23.

CAO, S., YAO, J., MCCABE, T.J., YAO, Q., KATUSIC, Z.S., SESSA, W.C. & SHAH, V. (2001). Direct interaction between endothelial nitric-oxide synthase and dynamin-2. Implications for nitric-oxide synthase function. *J Biol Chem*, **276,** 14249-56.

CARVAJAL, J.A., GERMAIN, A.M., HUIDOBRO-TORO, J.P. & WEINER, C.P. (2000). Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol*, **184,** 409-20.

CATTERALL, W.A., PEREZ-REYES, E., SNUTCH, T.P. & STRIESSNIG, J. (2005). International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol Rev*, **57,** 411-25.

CHAUHAN, S.D., NILSSON, H., AHLUWALIA, A. & HOBBS, A.J. (2003). Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor. *Proc Natl Acad Sci U S A*, **100,** 1426-31.

CHEN, G. & CHEUNG, D.W. (1997). Effect of K(+)-channel blockers on ACh-induced hyperpolarization and relaxation in mesenteric arteries. *Am J Physiol*, **272,** H2306-12.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br J Pharmacol*, **95,** 1165-74.

CHIDA, Y. & STEPTOE, A. (2010). Greater cardiovascular responses to laboratory mental stress are associated with poor subsequent cardiovascular risk status: a meta-analysis of prospective evidence. *Hypertension*, **55,** 1026-32.

CHOUDHARY, S., KUMAR, A., KALE, R.K., RAISZ, L.G. & PILBEAM, C.C. (2004). Extracellular calcium induces COX-2 in osteoblasts via a PKA pathway. *Biochem Biophys Res Commun*, **322,** 395-402.

CLAPHAM, D.E. & NEER, E.J. (1997). G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol*, **37,** 167-203.

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305,** 627-30.

COLEMAN, H.A., TARE, M. & PARKINGTON, H.C. (2002). Myoendothelial electrical coupling in arteries and arterioles and its implications for endothelium-derived hyperpolarizing factor. *Clin Exp Pharmacol Physiol*, **29,** 630-7.

CONTI, M.A. & ADELSTEIN, R.S. (1981). The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3':5' cAMP-dependent protein kinase. *J Biol Chem*, **256,** 3178-81.

CRANE, G.J., GALLAGHER, N., DORA, K.A. & GARLAND, C.J. (2003). Small- and intermediate-conductance calcium-activated K+ channels provide different facets of endothelium-dependent hyperpolarization in rat mesenteric artery. *J Physiol*, **553,** 183-9.

CRANE, G.J. & GARLAND, C.J. (2004). Thromboxane receptor stimulation associated with loss of SKCa activity and reduced EDHF responses in the rat isolated mesenteric artery. *Br J Pharmacol*, **142,** 43-50.

DAI, T., TIAN, Y., TOCCHETTI, C.G., KATORI, T., MURPHY, A.M., KASS, D.A., PAOLOCCI, N. & GAO, W.D. (2007). Nitroxyl increases force development in rat cardiac muscle. *J Physiol*, **580,** 951-60.

DALE, H.H. (1913). On the action of ergotoxine; with special reference to the existence of sympathetic vasodilators. *J Physiol*, **46,** 291-300.

DALY, C.J., ROSS, R.A., WHYTE, J., HENSTRIDGE, C.M., IRVING, A.J. & MCGRATH, J.C. (2010). Fluorescent ligand binding reveals heterogeneous distribution of adrenoceptors and 'cannabinoid-like' receptors in small arteries. *Br J Pharmacol*, **159,** 787-96.

DAVIS, K.A., SAMSON, S.E., HAMMEL, K.E., KISS, L., FULOP, F. & GROVER, A.K. (2009). Functional linkage of Na+-Ca2+-exchanger to sarco/endoplasmic reticulum Ca2+ pump in coronary artery: comparison of smooth muscle and endothelial cells. *J Cell Mol Med*, **13,** 1775-83.

DE BOER, S.F. & VAN DER GUGTEN, J. (1987). Daily variations in plasma noradrenaline, adrenaline and corticosterone concentrations in rats. *Physiol Behav*, **40,** 323-8.

DE WIT, C. & GRIFFITH, T.M. (2010). Connexins and gap junctions in the EDHF phenomenon and conducted vasomotor responses. *Pflugers Arch*, **459,** 897-914.

DELASHAW, J.B. & DULING, B.R. (1991). Heterogeneity in conducted arteriolar vasomotor response is agonist dependent. *Am J Physiol*, **260,** H1276-82.

DESSY, C., MONIOTTE, S., GHISDAL, P., HAVAUX, X., NOIRHOMME, P. & BALLIGAND, J.L. (2004). Endothelial beta3-adrenoceptors mediate vasorelaxation of human coronary microarteries through nitric oxide and endothelium-dependent hyperpolarization. *Circulation*, **110,** 948-54.

DESSY, C., SALIEZ, J., GHISDAL, P., DANEAU, G., LOBYSHEVA, II, FRERART, F., BELGE, C., JNAOUI, K., NOIRHOMME, P., FERON, O. & BALLIGAND, J.L. (2005). Endothelial beta3-adrenoreceptors mediate nitric oxide-dependent vasorelaxation of coronary microvessels in response to the third-generation beta-blocker nebivolol. *Circulation*, **112,** 1198-205.

DHARMASHANKAR, K. & WIDLANSKY, M.E. (2010). Vascular endothelial function and hypertension: insights and directions. *Curr Hypertens Rep*, **12,** 448-55.

DIERKS, E.A. & BURSTYN, J.N. (1996a). Nitric oxide (NO), the only nitrogen monoxide redox form capable of activating soluble guanylyl cyclase. *Biochem Pharmacol*, **51,** 1593-600.

DIERKS, E.A. & BURSTYN, J.N. (1996b). Nitric oxide ($NO^{\bullet}$), the only nitrogen monoxide redox form capable of activating soluble guanylyl cyclase. *Biochem Pharmacol*, **51,** 1593-600.

DIETZ, R., SCHOMIG, A., RASCHER, W., STRASSER, R., LUTH, J.B., GANTEN, U. & KUBLER, W. (1982). Contribution of the sympathetic nervous system to the hypertensive effect of a high sodium diet in stroke-prone spontaneously hypertensive rats. *Hypertension*, **4,** 773-81.

DIKALOVA, A.E., GONGORA, M.C., HARRISON, D.G., LAMBETH, J.D., DIKALOV, S. & GRIENDLING, K.K. (2010). Upregulation of Nox1 in vascular smooth muscle leads to impaired endothelium-dependent relaxation via eNOS uncoupling. *Am J Physiol Heart Circ Physiol*, **299,** H673-9.

DILLON, P.F., AKSOY, M.O., DRISKA, S.P. & MURPHY, R.A. (1981). Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. *Science*, **211,** 495-7.

DIMMELER, S., FLEMING, I., FISSLTHALER, B., HERMANN, C., BUSSE, R. & ZEIHER, A.M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*, **399,** 601-5.

DOCHERTY, J.R. (2010). Subtypes of functional alpha1-adrenoceptor. *Cell Mol Life Sci*, **67,** 405-17.

DORA, K.A. (2001). Cell-cell communication in the vessel wall. *Vasc Med*, **6,** 43-50.

DORA, K.A. (2010). Coordination of vasomotor responses by the endothelium. *Circ J*, **74,** 226-32.

DORA, K.A., DOYLE, M.P. & DULING, B.R. (1997). Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles. *Proc Natl Acad Sci U S A*, **94,** 6529-34.

DORA, K.A., GALLAGHER, N.T., MCNEISH, A. & GARLAND, C.J. (2008). Modulation of endothelial cell KCa3.1 channels during endothelium-derived hyperpolarizing factor signaling in mesenteric resistance arteries. *Circ Res*, **102,** 1247-55.

DORA, K.A., HINTON, J.M., WALKER, S.D. & GARLAND, C.J. (2000). An indirect influence of phenylephrine on the release of endothelium-derived vasodilators in rat small mesenteric artery. *Br J Pharmacol*, **129,** 381-7.

DORA, K.A., INGS, N.T. & GARLAND, C.J. (2002). K(Ca) channel blockers reveal hyperpolarization and relaxation to K+ in rat isolated mesenteric artery. *Am J Physiol Heart Circ Physiol*, **283,** H606-14.

DORA, K.A., MARTIN, P.E., CHAYTOR, A.T., EVANS, W.H., GARLAND, C.J. & GRIFFITH, T.M. (1999). Role of heterocellular Gap junctional communication in endothelium-dependent smooth muscle hyperpolarization: inhibition by a connexin-mimetic peptide. *Biochem Biophys Res Commun*, **254,** 27-31.

DORA, K.A., SANDOW, S.L., GALLAGHER, N.T., TAKANO, H., RUMMERY, N.M., HILL, C.E. & GARLAND, C.J. (2003a). Myoendothelial gap junctions may provide the pathway for EDHF in mouse mesenteric artery. *J Vasc Res*, **40,** 480-90.

DORA, K.A., XIA, J. & DULING, B.R. (2003b). Endothelial cell signaling during conducted vasomotor responses. *Am J Physiol Heart Circ Physiol*, **285,** H119-26.

DOUGHTY, J.M., PLANE, F. & LANGTON, P.D. (1999). Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. *Am J Physiol*, **276,** H1107-12.

DOYLE, M.P. & DULING, B.R. (1997). Acetylcholine induces conducted vasodilation by nitric oxide-dependent and -independent mechanisms. *Am J Physiol*, **272,** H1364-71.

DREXLER, H., HAYOZ, D., MUNZEL, T., JUST, H., ZELIS, R. & BRUNNER, H.R. (1994). Endothelial dysfunction in chronic heart failure. Experimental and clinical studies. *Arzneimittelforschung*, **44,** 455-8.

DUDZINSKI, D.M., IGARASHI, J., GREIF, D. & MICHEL, T. (2006). The regulation and pharmacology of endothelial nitric oxide synthase. *Annu Rev Pharmacol Toxicol*, **46,** 235-76.

DULING, B.R., GORE, R.W., DACEY, R.G., JR. & DAMON, D.N. (1981). Methods for isolation, cannulation, and in vitro study of single microvessels. *Am J Physiol*, **241,** H108-116.

DUNN, W.R., BROCK, J.A. & HARDY, T.A. (1999). Electrochemical and electrophysiological characterization of neurotransmitter release from sympathetic nerves supplying rat mesenteric arteries. *Br J Pharmacol*, **128,** 174-80.

DUQUESNES, N., DERANGEON, M., METRICH, M., LUCAS, A., MATEO, P., LI, L., MOREL, E., LEZOUALC'H, F. & CROZATIER, B. (2010). Epac stimulation induces rapid increases in connexin43 phosphorylation and function without preconditioning effect. *Pflugers Arch*, **460,** 731-41.

DUSTING, G.J., MONCADA, S. & VANE, J.R. (1977). Prostacyclin (PGI2) is a weak contractor of coronary arteries of the pig. *Eur J Pharmacol*, **45,** 301-4.

EARLEY, S., HEPPNER, T.J., NELSON, M.T. & BRAYDEN, J.E. (2005). TRPV4 forms a novel Ca2+ signaling complex with ryanodine receptors and BKCa channels. *Circ Res*, **97,** 1270-9.

EDVINSSON, L. & OWMAN, C. (1974). Pharmacological characterization of adrenergic alpha and beta receptors mediating the vasomotor responses of cerebral arteries in vitro. *Circ Res*, **35,** 835-49.

EDWARDS, G., DORA, K.A., GARDENER, M.J., GARLAND, C.J. & WESTON, A.H. (1998). K+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature*, **396,** 269-72.

EGLEN, R.M. (2006). Muscarinic receptor subtypes in neuronal and non-neuronal cholinergic function. *Auton Autacoid Pharmacol*, **26,** 219-33.

ELLIS, A., LI, C.G. & RAND, M.J. (2000). Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta. *Br J Pharmacol*, **129,** 315-22.

EMERSON, G.G., NEILD, T.O. & SEGAL, S.S. (2002). Conduction of hyperpolarization along hamster feed arteries: augmentation by acetylcholine. *Am J Physiol Heart Circ Physiol*, **283,** H102-9.

EMERSON, G.G. & SEGAL, S.S. (2001). Electrical activation of endothelium evokes vasodilation and hyperpolarization along hamster feed arteries. *Am J Physiol Heart Circ Physiol*, **280,** H160-7.

EMERSON, G.G. & SEGAL, S.S. (2000). Endothelial cell pathway for conduction of hyperpolarization and vasodilation along hamster feed artery. *Circ Res*, **86,** 94-100.

ENOURI, S., MONTEITH, G. & JOHNSON, R. (2010). Characteristics of myogenic reactivity in isolated rat mesenteric veins. *Am J Physiol Regul Integr Comp Physiol*, **300,** R470-8.

ETO, M., SENBA, S., MORITA, F. & YAZAWA, M. (1997). Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: its specific localization in smooth muscle. *FEBS Lett*, **410,** 356-60.

ETTER, E.F., ETO, M., WARDLE, R.L., BRAUTIGAN, D.L. & MURPHY, R.A. (2001). Activation of myosin light chain phosphatase in intact arterial smooth muscle during nitric oxide-induced relaxation. *J Biol Chem*, **276,** 34681-5.

FAVALORO, J.L. & KEMP-HARPER, B.K. (2007). The nitroxyl anion (HNO) is a potent dilator of rat coronary vasculature. *Cardiovasc Res*, **73,** 587-96.

FAVALORO, J.L. & KEMP-HARPER, B.K. (2009). Redox variants of nitric oxide (NO* and HNO) elicit vasorelaxation of resistance arteries via distinct mechanisms. *Am J Physiol Heart Circ Physiol*.

FEELISCH, M. (1993). Biotransformation to nitric oxide of organic nitrates in comparison to other nitrovasodilators. *Eur Heart J*, **14 Suppl I,** 123-32.

FEELISCH, M., TE POEL, M., ZAMORA, R., DEUSSEN, A. & MONCADA, S. (1994). Understanding the controversy over the identity of EDRF. *Nature*, **368,** 62-5.

FELETOU, M., HUANG, Y. & VANHOUTTE, P.M. (2010a). Vasoconstrictor prostanoids. *Pflugers Arch*, **459,** 941-50.

FELETOU, M., KOHLER, R. & VANHOUTTE, P.M. (2010b). Endothelium-derived vasoactive factors and hypertension: possible roles in pathogenesis and as treatment targets. *Curr Hypertens Rep*, **12,** 267-75.

FELETOU, M., VERBEUREN, T.J. & VANHOUTTE, P.M. (2009). Endothelium-dependent contractions in SHR: a tale of prostanoid TP and IP receptors. *Br J Pharmacol*, **156,** 563-74.

FERRO, A., COASH, M., YAMAMOTO, T., ROB, J., JI, Y. & QUEEN, L. (2004). Nitric oxide-dependent beta2-adrenergic dilatation of rat aorta is mediated through activation of both protein kinase A and Akt. *Br J Pharmacol*, **143,** 397-403.

FETALVERO, K.M., MARTIN, K.A. & HWA, J. (2007). Cardioprotective prostacyclin signaling in vascular smooth muscle. *Prostaglandins Other Lipid Mediat*, **82,** 109-18.

FIGUEROA, X.F., CHEN, C.C., CAMPBELL, K.P., DAMON, D.N., DAY, K.H., RAMOS, S. & DULING, B.R. (2007). Are voltage-dependent ion channels involved in the endothelial cell control of vasomotor tone? *Am J Physiol Heart Circ Physiol*, **293,** H1371-83.

FIGUEROA, X.F. & DULING, B.R. (2009a). Gap junctions in the control of vascular function. *Antioxid Redox Signal*, **11,** 251-66.

FIGUEROA, X.F., GONZALEZ, D.R., MARTINEZ, A.D., DURAN, W.N. & BORIC, M.P. (2002). ACh-induced endothelial NO synthase translocation, NO release and vasodilatation in the hamster microcirculation in vivo. *J Physiol*, **544,** 883-96.

FIGUEROA, X.F., POBLETE, I., FERNANDEZ, R., PEDEMONTE, C., CORTES, V. & HUIDOBRO-TORO, J.P. (2009b). NO production and eNOS phosphorylation

induced by adrenaline through the activation of {beta}-adrenoceptors. *Am J Physiol Heart Circ Physiol*.

FIGUEROA, X.F., POBLETE, M.I., BORIC, M.P., MENDIZABAL, V.E., ADLER-GRASCHINSKY, E. & HUIDOBRO-TORO, J.P. (2001). Clonidine-induced nitric oxide-dependent vasorelaxation mediated by endothelial alpha(2)-adrenoceptor activation. *Br J Pharmacol*, **134,** 957-68.

FILIPPI, S., PARENTI, A., DONNINI, S., GRANGER, H.J., FAZZINI, A. & LEDDA, F. (2001). alpha(1D)-adrenoceptors cause endothelium-dependent vasodilatation in the rat mesenteric vascular bed. *J Pharmacol Exp Ther*, **296,** 869-75.

FLAA, A., EIDE, I.K., KJELDSEN, S.E. & ROSTRUP, M. (2008). Sympathoadrenal stress reactivity is a predictor of future blood pressure: an 18-year follow-up study. *Hypertension*, **52,** 336-41.

FLEMING, I. (2010). Molecular mechanisms underlying the activation of eNOS. *Pflugers Arch*, **459,** 793-806.

FLEMING, I. & BUSSE, R. (1999a). NO: the primary EDRF. *J Mol Cell Cardiol*, **31,** 5-14.

FLEMING, I., FISSLTHALER, B., DIMMELER, S., KEMP, B.E. & BUSSE, R. (2001). Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res*, **88,** E68-75.

FLEMING, I., SCHERMER, B., POPP, R. & BUSSE, R. (1999b). Inhibition of the production of endothelium-derived hyperpolarizing factor by cannabinoid receptor agonists. *Br J Pharmacol*, **126,** 949-60.

FLORAS, J.S. (1992). Epinephrine and the genesis of hypertension. *Hypertension*, **19,** 1-18.

FORMEY, A., BUSCEMI, L., BOITTIN, F.X., BENY, J.L. & MEISTER, J.J. (2011). Identification and functional response of interstitial Cajal-like cells from rat mesenteric artery. *Cell Tissue Res*, **343,** 509-19.

FREITAS, M.R., ETO, M., KIRKBRIDE, J.A., SCHOTT, C., SASSARD, J. & STOCLET, J.C. (2009). Y27632, a Rho-activated kinase inhibitor, normalizes dysregulation in alpha1-adrenergic receptor-induced contraction of Lyon hypertensive rat artery smooth muscle. *Fundam Clin Pharmacol*, **23,** 169-78.

FRIEDEMANN, M.N., ROBINSON, S.W. & GERHARDT, G.A. (1996). o-Phenylenediamine-modified carbon fiber electrodes for the detection of nitric oxide. *Anal Chem*, **68,** 2621-8.

FUJII, K., ONAKA, U., GOTO, K., ABE, I. & FUJISHIMA, M. (1999). Impaired isoproterenol-induced hyperpolarization in isolated mesenteric arteries of aged rats. *Hypertension*, **34,** 222-8.

FUJII, T., YAMADA, S., YAMAGUCHI, N., FUJIMOTO, K., SUZUKI, T. & KAWASHIMA, K. (1995). Species differences in the concentration of acetylcholine, a neurotransmitter, in whole blood and plasma. *Neurosci Lett*, **201,** 207-10.

FUJIMOTO, S. & MATSUDA, T. (1991). M3 cholinoceptors and P2y purinoceptors mediating relaxation of arteries in spontaneously hypertensive rats at prehypertensive stages. *Eur J Pharmacol*, **202,** 9-15.

FUJIWARA, T. & ANGUS, J.A. (1996). Analysis of relaxation and repolarization mechanisms of nicorandil in rat mesenteric artery. *Br J Pharmacol*, **119,** 1549-56.

FUKAO, M., HATTORI, Y., KANNO, M., SAKUMA, I. & KITABATAKE, A. (1997). Sources of Ca2+ in relation to generation of acetylcholine-induced endothelium-dependent hyperpolarization in rat mesenteric artery. *Br J Pharmacol*, **120,** 1328-34.

FUKUDA, N., IZUMI, Y., SOMA, M., WATANABE, Y., WATANABE, M. & HATANO, M. (1992). Effects of L-NG-monomethyl arginine on the cyclic GMP formations in rat mesenteric arteries. *Jpn J Pharmacol*, **58,** 55-60.

FUKUTO, J.M., HOBBS, A.J. & IGNARRO, L.J. (1993). Conversion of nitroxyl (HNO) to nitric oxide (NO) in biological systems: the role of physiological oxidants and relevance to the biological activity of HNO. *Biochem Biophys Res Commun*, **196,** 707-13.

FURCHGOTT, R.F. & JOTHIANANDAN, D. (1991). Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels*, **28,** 52-61.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288,** 373-6.

FURUKAWA, K., TAWADA, Y. & SHIGEKAWA, M. (1988). Regulation of the plasma membrane Ca2+ pump by cyclic nucleotides in cultured vascular smooth muscle cells. *J Biol Chem*, **263,** 8058-65.

GALETTA, F., FRANZONI, F., BERNINI, G., POUPAK, F., CARPI, A., CINI, G., TOCCHINI, L., ANTONELLI, A. & SANTORO, G. (2010). Cardiovascular complications in patients with pheochromocytoma: a mini-review. *Biomed Pharmacother*, **64,** 505-9.

GAO, Y.J. & LEE, R.M. (2001). Hydrogen peroxide induces a greater contraction in mesenteric arteries of spontaneously hypertensive rats through thromboxane A(2) production. *Br J Pharmacol*, **134,** 1639-46.

GAO, Y.J., ZHANG, Y., HIROTA, S., JANSSEN, L.J. & LEE, R.M. (2004). Vascular relaxation response to hydrogen peroxide is impaired in hypertension. *Br J Pharmacol*, **142,** 143-9.

GARCIA-REDONDO, A.B., BRIONES, A.M., BELTRAN, A.E., ALONSO, M.J., SIMONSEN, U. & SALAICES, M. (2009). Hypertension increases contractile responses to hydrogen peroxide in resistance arteries through increased thromboxane A2, Ca2+, and superoxide anion levels. *J Pharmacol Exp Ther*, **328,** 19-27.

GARLAND, C.J. & DORA, K.A. (2008). Evidence against C-type natriuretic peptide as an arterial 'EDHF'. *Br J Pharmacol*, **153,** 4-5.

GARLAND, C.J., HILEY, C.R. & DORA, K.A. (2010a). EDHF: spreading the influence of the endothelium. *Br J Pharmacol*.

GARLAND, C.J. & MCPHERSON, G.A. (1992a). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br J Pharmacol*, **105,** 429-35.

GARLAND, C.J., YAROVA, P., JIMENEZ-ALTAYO, F. & DORA, K.A. (2011). Vascular hyperpolarization to beta-adrenoceptor agonists evokes spreading dilatation in rat isolated mesenteric arteries. *Br J Pharmacol*.

GARLAND, C.J., YAROVA, P., JIMENEZ-ALTAYO, F. & DORA, K.A. (2010b). Vascular hyperpolarization to beta-adrenoceptor agonists evokes spreading dilatation in rat isolated mesenteric arteries. *Br J Pharmacol*.

GARLAND, J.G. & MCPHERSON, G.A. (1992b). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br J Pharmacol*, **105,** 429-35.

GATENBECK, L., JOHANSSON, B., STROMBERG, L. & SVENSSON, M. (1987). Genital blood flow in male rats subjected to stress stimuli. *Urol Res*, **15,** 297-301.

GAYEN, J.R., ZHANG, K., RAMACHANDRARAO, S.P., MAHATA, M., CHEN, Y., KIM, H.S., NAVIAUX, R.K., SHARMA, K., MAHATA, S.K. & O'CONNOR, D.T. (2010). Role of reactive oxygen species in hyperadrenergic hypertension: biochemical,

physiological, and pharmacological evidence from targeted ablation of the chromogranin a (Chga) gene. *Circ Cardiovasc Genet*, **3,** 414-25.
GILBERT, P., TREMBLAY, J. & THORIN, E. (2001). Endothelium-derived endothelin-1 reduces cerebral artery sensitivity to nitric oxide by a protein kinase C-independent pathway. *Stroke*, **32,** 2351-5.
GLOERICH, M. & BOS, J.L. (2010). Epac: defining a new mechanism for cAMP action. *Annu Rev Pharmacol Toxicol*, **50,** 355-75.
GOKINA, N.I., KNOT, H.J., NELSON, M.T. & OSOL, G. (1999). Increased Ca2+ sensitivity as a key mechanism of PKC-induced constriction in pressurized cerebral arteries. *Am J Physiol*, **277,** H1178-88.
GOLDSTEIN, D.S. (1983). Plasma catecholamines and essential hypertension. An analytical review. *Hypertension*, **5,** 86-99.
GOMEZ, E., SCHWENDEMANN, C., ROGER, S., SIMONET, S., PAYSANT, J., COURCHAY, C., VERBEUREN, T.J. & FELETOU, M. (2008). Aging and prostacyclin responses in aorta and platelets from WKY and SHR rats. *Am J Physiol Heart Circ Physiol*, **295,** H2198-211.
GOTO, K., RUMMERY, N.M., GRAYSON, T.H. & HILL, C.E. (2004). Attenuation of conducted vasodilatation in rat mesenteric arteries during hypertension: role of inwardly rectifying potassium channels. *J Physiol*, **561,** 215-31.
GRASSI, G., SERAVALLE, G. & QUARTI-TREVANO, F. (2010). The 'neuroadrenergic hypothesis' in hypertension: current evidence. *Exp Physiol*, **95,** 581-6.
GRAVES, J. & POSTON, L. (1993). Beta-adrenoceptor agonist mediated relaxation of rat isolated resistance arteries: a role for the endothelium and nitric oxide. *Br J Pharmacol*, **108,** 631-7.
GRAYSON, T.H., HADDOCK, R.E., MURRAY, T.P., WOJCIKIEWICZ, R.J. & HILL, C.E. (2004). Inositol 1,4,5-trisphosphate receptor subtypes are differentially distributed between smooth muscle and endothelial layers of rat arteries. *Cell Calcium*, **36,** 447-58.
GREIF, D.M., SACKS, D.B. & MICHEL, T. (2004). Calmodulin phosphorylation and modulation of endothelial nitric oxide synthase catalysis. *Proc Natl Acad Sci U S A*, **101,** 1165-70.
GRGIC, I., KAISTHA, B.P., HOYER, J. & KOHLER, R. (2009). Endothelial Ca+-activated K+ channels in normal and impaired EDHF-dilator responses--relevance to cardiovascular pathologies and drug discovery. *Br J Pharmacol*, **157,** 509-26.
GRIFFITH, T.M., CHAYTOR, A.T., TAYLOR, H.J., GIDDINGS, B.D. & EDWARDS, D.H. (2002). cAMP facilitates EDHF-type relaxations in conduit arteries by enhancing electrotonic conduction via gap junctions. *Proc Natl Acad Sci U S A*, **99,** 6392-7.
GROS, R., AFROZE, T., YOU, X.M., KABIR, G., VAN WERT, R., KALAIR, W., HOQUE, A.E., MUNGRUE, I.N. & HUSAIN, M. (2003). Plasma membrane calcium ATPase overexpression in arterial smooth muscle increases vasomotor responsiveness and blood pressure. *Circ Res*, **93,** 614-21.
GRUEB, M., BARTZ-SCHMIDT, K.U. & ROHRBACH, J.M. (2008). Adrenergic Regulation of cAMP/Protein Kinase A Pathway in Corneal Epithelium and Endothelium. *Ophthalmic Res*, **40,** 322-328.
GUDERMANN, T., HOFMANN, T., MEDEROS Y SCHNITZLER, M. & DIETRICH, A. (2004). Activation, subunit composition and physiological relevance of DAG-sensitive TRPC proteins. *Novartis Found Symp*, **258,** 103-18; discussion 118-22, 155-9, 263-6.

GUIMARAES, S. & MOURA, D. (2001). Vascular adrenoceptors: an update. *Pharmacol Rev*, **53,** 319-56.
GUPTA, S. & WRIGHT, H.M. (2008). Nebivolol: a highly selective beta1-adrenergic receptor blocker that causes vasodilation by increasing nitric oxide. *Cardiovasc Ther*, **26,** 189-202.
GURDAL, H., FRIEDMAN, E. & JOHNSON, M.D. (1995). Beta-adrenoceptor-G alpha S coupling decreases with age in rat aorta. *Mol Pharmacol*, **47,** 772-8.
GUSTAFSSON, H. (1993). Vasomotion and underlying mechanisms in small arteries. An in vitro study of rat blood vessels. *Acta Physiol Scand Suppl*, **614,** 1-44.
HAI, C.M. & MURPHY, R.A. (1989). Ca2+, crossbridge phosphorylation, and contraction. *Annu Rev Physiol*, **51,** 285-98.
HARDIE, R.C. (2007). TRP channels and lipids: from Drosophila to mammalian physiology. *J Physiol*, **578,** 9-24.
HARMS, H.H., ZAAGSMA, J. & VAN DER WAL, B. (1974). Beta-adrenoceptor studies. III. On the beta-adrenoceptors in rat adipose tissue. *Eur J Pharmacol*, **25,** 87-91.
HARPER, M.T., MOLKENTIN, J.D. & POOLE, A.W. (2010). Protein kinase C alpha enhances sodium-calcium exchange during store-operated calcium entry in mouse platelets. *Cell Calcium*, **48,** 333-40.
HASHIGUCHI, H., YE, S.H., ROSS-CISNEROS, F. & ALEXANDER, N. (1997). Central nitric oxide donors attenuate cardiovascular and central norepinephrine responses to stress. *Am J Physiol*, **272,** R1447-53.
HASHIMOTO, A., MIYAKODA, G., HIROSE, Y. & MORI, T. (2006). Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism. *Atherosclerosis*, **189,** 350-7.
HAUG, S.J., WELSH, D.G. & SEGAL, S.S. (2003). Sympathetic nerves inhibit conducted vasodilatation along feed arteries during passive stretch of hamster skeletal muscle. *J Physiol*, **552,** 273-82.
HEFFERNAN, K.S., SURYADEVARA, R., PATVARDHAN, E.A., MOONEY, P., KARAS, R.H. & KUVIN, J.T. (2011). Effect of Atenolol vs Metoprolol Succinate on Vascular Function in Patients With Hypertension. *Clin Cardiol*, **34,** 39-44.
HEYMAN, N.S., KURJIAKA, D.T., EK VITORIN, J.F. & BURT, J.M. (2009). Regulation of gap junctional charge selectivity in cells coexpressing connexin 40 and connexin 43. *Am J Physiol Heart Circ Physiol*, **297,** H450-9.
HIGASHI, Y., SASAKI, S., NAKAGAWA, K., KIMURA, M., SASAKI, S., NOMA, K., MATSUURA, H., HARA, K., GOTO, C., OSHIMA, T. & CHAYAMA, K. (2002). Excess norepinephrine impairs both endothelium-dependent and -independent vasodilation in patients with pheochromocytoma. *Hypertension*, **39,** 513-8.
HILL, A.J., HINTON, J.M., CHENG, H., GAO, Z., BATES, D.O., HANCOX, J.C., LANGTON, P.D. & JAMES, A.F. (2006). A TRPC-like non-selective cation current activated by alpha 1-adrenoceptors in rat mesenteric artery smooth muscle cells. *Cell Calcium*, **40,** 29-40.
HIRATA, K., KURODA, R., SAKODA, T., KATAYAMA, M., INOUE, N., SUEMATSU, M., KAWASHIMA, S. & YOKOYAMA, M. (1995). Inhibition of endothelial nitric oxide synthase activity by protein kinase C. *Hypertension*, **25,** 180-5.
HIRATA, T., USHIKUBI, F., KAKIZUKA, A., OKUMA, M. & NARUMIYA, S. (1996). Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J Clin Invest*, **97,** 949-56.

HOBBS, A., FOSTER, P., PRESCOTT, C., SCOTLAND, R. & AHLUWALIA, A. (2004). Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide. *Circulation*, **110,** 1231-5.

HOBBS, A.J., FUKUTO, J.M. & IGNARRO, L.J. (1994). Formation of free nitric oxide from l-arginine by nitric oxide synthase: direct enhancement of generation by superoxide dismutase. *Proc Natl Acad Sci U S A*, **91,** 10992-6.

HOLMAN, M.E., KASBY, C.B., SUTHERS, M.B. & WILSON, J.A. (1968). Some properties of the smooth muscle of rabbit portal vein. *J Physiol*, **196,** 111-32.

HORIGUCHI, S., WATANABE, J., KATO, H., BABA, S., SHINOZAKI, T., MIURA, M., FUKUCHI, M., KAGAYA, Y. & SHIRATO, K. (2001). Contribution of Na+/Ca2+ exchanger to the regulation of myogenic tone in isolated rat small arteries. *Acta Physiol Scand*, **173,** 167-73.

HROMETZ, S.L., EDELMANN, S.E., MCCUNE, D.F., OLGES, J.R., HADLEY, R.W., PEREZ, D.M. & PIASCIK, M.T. (1999). Expression of multiple alpha1-adrenoceptors on vascular smooth muscle: correlation with the regulation of contraction. *J Pharmacol Exp Ther*, **290,** 452-63.

HUANG, Y., KWOK, K.H., CHAN, N.W., LAU, C.W. & CHEN, Z.Y. (1998). Role of endothelium and K+ channels in dobutamine-induced relaxation in rat mesenteric artery. *Clin Exp Pharmacol Physiol*, **25,** 405-11.

HUGHES, M.N. (1999). Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. *Biochim Biophys Acta*, **1411,** 263-72.

HUNT, J.A., MERRITT, J.E., MACDERMOT, J. & KEEN, M. (1992). Characterization of the thromboxane receptor mediating prostacyclin release from cultured endothelial cells. *Biochem Pharmacol*, **43,** 1747-52.

HUSSAIN, M.B. & MARSHALL, I. (2000). Alpha(1)-adrenoceptor subtypes mediating contractions of the rat mesenteric artery. *Eur J Pharmacol*, **395,** 69-76.

HUTCHINSON, D.S., EVANS, B.A. & SUMMERS, R.J. (2001). beta(1)-Adrenoceptors compensate for beta(3)-adrenoceptors in ileum from beta(3)-adrenoceptor knock-out mice. *Br J Pharmacol*, **132,** 433-42.

IGNARRO, L.J., BYRNS, R.E., BUGA, G.M. & WOOD, K.S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ Res*, **61,** 866-79.

INOGUCHI, T., UEDA, F., UMEDA, F., YAMASHITA, T. & NAWATA, H. (1995). Inhibition of intercellular communication via gap junction in cultured aortic endothelial cells by elevated glucose and phorbol ester. *Biochem Biophys Res Commun*, **208,** 492-7.

IRVINE, J.C., FAVALORO, J.L. & KEMP-HARPER, B.K. (2003a). NO- activates soluble guanylate cyclase and Kv channels to vasodilate resistance arteries. *Hypertension*, **41,** 1301-7.

IRVINE, J.C., FAVALORO, J.L. & KEMP-HARPER, B.K. (2003b). NO^{-} activates soluble guanylate cyclase and K_V channels to vasodilate resistance arteries. *Hypertension*, **41,** 1301-7.

IRVINE, J.C., FAVALORO, J.L., WIDDOP, R.E. & KEMP-HARPER, B.K. (2007). Nitroxyl anion donor, Angeli's salt, does not develop tolerance in rat isolated aortae. *Hypertension*, **49,** 885-92.

IRVINE, J.C., RITCHIE, R.H., FAVALORO, J.L., ANDREWS, K.L., WIDDOP, R.E. & KEMP-HARPER, B.K. (2008). Nitroxyl (HNO): the Cinderella of the nitric oxide story. *Trends Pharmacol Sci*.

IWATANI, Y., KOSUGI, K., ISOBE-OKU, S., ATAGI, S., KITAMURA, Y. & KAWASAKI, H. (2008). Endothelium removal augments endothelium-independent vasodilatation in rat mesenteric vascular bed. *Br J Pharmacol*, **154,** 32-40.

IWATANI, Y., NUMA, H., ATAGI, S., TAKAYAMA, F., MIO, M. & KAWASAKI, H. (2007). [Mechanisms underlying enhanced vasodilator responses to various vasodilator agents following endothelium removal in rat mesenteric resistance arteries]. *Yakugaku Zasshi*, **127,** 729-33.

JAGGAR, J.H., WELLMAN, G.C., HEPPNER, T.J., PORTER, V.A., PEREZ, G.J., GOLLASCH, M., KLEPPISCH, T., RUBART, M., STEVENSON, A.S., LEDERER, W.J., KNOT, H.J., BONEV, A.D. & NELSON, M.T. (1998). Ca2+ channels, ryanodine receptors and Ca(2+)-activated K+ channels: a functional unit for regulating arterial tone. *Acta Physiol Scand*, **164,** 577-87.

JANTZI, M.C., BRETT, S.E., JACKSON, W.F., CORTELING, R., VIGMOND, E.J. & WELSH, D.G. (2006). Inward rectifying potassium channels facilitate cell-to-cell communication in hamster retractor muscle feed arteries. *Am J Physiol Heart Circ Physiol*, **291,** H1319-28.

JENSEN, L.J., SALOMONSSON, M., JENSEN, B.L. & HOLSTEIN-RATHLOU, N.H. (2004). Depolarization-induced calcium influx in rat mesenteric small arterioles is mediated exclusively via mibefradil-sensitive calcium channels. *Br J Pharmacol*, **142,** 709-18.

JIN, X., SATOH-OTONASHI, Y., ZAMAMI, Y., KOYAMA, T., SUN, P., KITAMURA, Y. & KAWASAKI, H. (2008). Characterization of the inhibitory effect of vascular endothelium on agonist-induced vasoconstriction in rat mesenteric resistance arteries. *J Pharmacol Sci*, **108,** 95-103.

JOHNSEN, D.D., KACIMI, R., ANDERSON, B.E., THOMAS, T.A., SAID, S. & GERDES, A.M. (2005). Protein kinase C isozymes in hypertension and hypertrophy: insight from SHHF rat hearts. *Mol Cell Biochem*, **270,** 63-9.

KANG, K.B., RAJANAYAGAM, M.A., VAN DER ZYPP, A. & MAJEWSKI, H. (2007). A role for cyclooxygenase in aging-related changes of beta-adrenoceptor-mediated relaxation in rat aortas. *Naunyn Schmiedebergs Arch Pharmacol*, **375,** 273-81.

KANSUI, Y., FUJII, K., NAKAMURA, K., GOTO, K., ONIKI, H., ABE, I., SHIBATA, Y. & IIDA, M. (2004). Angiotensin II receptor blockade corrects altered expression of gap junctions in vascular endothelial cells from hypertensive rats. *Am J Physiol Heart Circ Physiol*, **287,** H216-24.

KANSUI, Y., GARLAND, C.J. & DORA, K.A. (2008). Enhanced spontaneous Ca2+ events in endothelial cells reflect signalling through myoendothelial gap junctions in pressurized mesenteric arteries. *Cell Calcium*, **44,** 135-46.

KATADA, J. & MAJIMA, M. (2002). AT(2) receptor-dependent vasodilation is mediated by activation of vascular kinin generation under flow conditions. *Br J Pharmacol*, **136,** 484-91.

KATO, E., LIPTAK, L., SHUJAA, N., MATYUS, P., GYIRES, K. & RONAI, A.Z. (2008). alpha2B-adrenoceptor agonist ST-91 antagonizes beta2-adrenoceptor-mediated relaxation in rat mesenteric artery rings. *Eur J Pharmacol*, **580,** 361-5.

KATO, H., FUKAMI, K., SHIBASAKI, F., HOMMA, Y. & TAKENAWA, T. (1992). Enhancement of phospholipase C delta 1 activity in the aortas of spontaneously hypertensive rats. *J Biol Chem*, **267,** 6483-7.

KAUMANN, A.J., ENGELHARDT, S., HEIN, L., MOLENAAR, P. & LOHSE, M. (2001). Abolition of (-)-CGP 12177-evoked cardiostimulation in double beta1/beta2-adrenoceptor knockout mice. Obligatory role of beta1-adrenoceptors for putative

beta4-adrenoceptor pharmacology. *Naunyn Schmiedebergs Arch Pharmacol*, **363,** 87-93.

KAUPP, U.B. & SEIFERT, R. (2002). Cyclic nucleotide-gated ion channels. *Physiol Rev*, **82,** 769-824.

KAWAMURA, K., ANDO, K. & TAKEBAYASHI, S. (1989). Perivascular innervation of the mesenteric artery in spontaneously hypertensive rats. *Hypertension*, **14,** 660-5.

KEDZIORA-KORNATOWSKA, K., CZUCZEJKO, J., SZEWCZYK-GOLEC, K., MOTYL, J., SZADUJKIS-SZADURSKI, L., KORNATOWSKI, T., PAWLUK, H. & KEDZIORA, J. (2006). Effects of perindopril and hydrochlorothiazide on selected indices of oxidative stress in the blood of elderly patients with essential hypertension. *Clin Exp Pharmacol Physiol*, **33,** 751-6.

KEMP, B.K. & MCPHERSON, G.A. (1998). Interspecies differences in thromboxane A2 receptors are distinguished by glibenclamide. *Eur J Pharmacol*, **354,** 173-8.

KLEINBONGARD, P., DEJAM, A., LAUER, T., JAX, T., KERBER, S., GHARINI, P., BALZER, J., ZOTZ, R.B., SCHARF, R.E., WILLERS, R., SCHECHTER, A.N., FEELISCH, M. & KELM, M. (2006). Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. *Free Radic Biol Med*, **40,** 295-302.

KLEMOLA, R., HUTTUNEN, P., LAINE, M., WECKSTROM, M. & HIRVONEN, J. (1999). Catecholamines in pericardial fluid of normotensive, spontaneously hypertensive and reserpine-treated rats. *Acta Physiol Scand*, **165,** 293-7.

KNOCK, G.A., SNETKOV, V.A., SHAIFTA, Y., CONNOLLY, M., DRNDARSKI, S., NOAH, A., POURMAHRAM, G.E., BECKER, S., AARONSON, P.I. & WARD, J.P. (2009). Superoxide constricts rat pulmonary arteries via Rho-kinase-mediated Ca(2+) sensitization. *Free Radic Biol Med*, **46,** 633-42.

KO, E.A., HAN, J., JUNG, I.D. & PARK, W.S. (2008). Physiological roles of K+ channels in vascular smooth muscle cells. *J Smooth Muscle Res*, **44,** 65-81.

KO, E.A., PARK, W.S., FIRTH, A.L., KIM, N., YUAN, J.X. & HAN, J. (2010). Pathophysiology of voltage-gated K+ channels in vascular smooth muscle cells: modulation by protein kinases. *Prog Biophys Mol Biol*, **103,** 95-101.

KOLLER, A. & HUANG, A. (1994). Impaired nitric oxide-mediated flow-induced dilation in arterioles of spontaneously hypertensive rats. *Circ Res*, **74,** 416-21.

KOMALAVILAS, P. & LINCOLN, T.M. (1996). Phosphorylation of the inositol 1,4,5-trisphosphate receptor. Cyclic GMP-dependent protein kinase mediates cAMP and cGMP dependent phosphorylation in the intact rat aorta. *J Biol Chem*, **271,** 21933-8.

KOU, R. & MICHEL, T. (2007). Epinephrine regulation of the endothelial nitric-oxide synthase: roles of RAC1 and beta3-adrenergic receptors in endothelial NO signaling. *J Biol Chem*, **282,** 32719-29.

KOZLOWSKA, H., SZYMSKA, U., SCHLICKER, E. & MALINOWSKA, B. (2003). Atypical beta-adrenoceptors, different from beta 3-adrenoceptors and probably from the low-affinity state of beta 1-adrenoceptors, relax the rat isolated mesenteric artery. *Br J Pharmacol*, **140,** 3-12.

KROTOVA, K.Y., ZHARIKOV, S.I. & BLOCK, E.R. (2003). Classical isoforms of PKC as regulators of CAT-1 transporter activity in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol*, **284,** L1037-44.

KRUSE, L.S., SANDHOLDT, N.T., GAMMELTOFT, S., OLESEN, J. & KRUUSE, C. (2006). Phosphodiesterase 3 and 5 and cyclic nucleotide-gated ion channel expression in rat trigeminovascular system. *Neurosci Lett*, **404,** 202-7.

KUBO, M., QUAYLE, J.M. & STANDEN, N.B. (1997). Angiotensin II inhibition of ATP-sensitive K+ currents in rat arterial smooth muscle cells through protein kinase C. *J Physiol*, **503 (Pt 3),** 489-96.

KUBO, S.H., RECTOR, T.S., BANK, A.J., WILLIAMS, R.E. & HEIFETZ, S.M. (1991). Endothelium-dependent vasodilation is attenuated in patients with heart failure. *Circulation*, **84,** 1589-96.

KUKLINSKA, A.M., MROCZKO, B., MUSIAL, W.J., SAWICKI, R., KAMINSKI, K., USOWICZ-SZARYNSKA, M. & SZMITKOWSKI, M. (2010). Endothelial dysfunction and sympathetic nervous system activation in young patients with essential arterial hypertension and without hypercholesterolaemia. *Acta Cardiol*, **65,** 535-40.

KUKOVETZ, W.R., HOLZMANN, S., WURM, A. & POCH, G. (1979). Prostacyclin increases cAMP in coronary arteries. *J Cyclic Nucleotide Res*, **5,** 469-76.

KWAN, H.Y., CHENG, K.T., MA, Y., HUANG, Y., TANG, N.L., YU, S. & YAO, X. (2010). CNGA2 contributes to ATP-induced noncapacitative Ca2+ influx in vascular endothelial cells. *J Vasc Res*, **47,** 148-56.

LA, M., LI, C.G. & RAND, M.J. (1996). Comparison of the effects of hydroxocobalamin and oxyhaemoglobin on responses to NO, EDRF and the nitrergic transmitter. *Br J Pharmacol*, **117,** 805-10.

LAGAUD, G.J., RANDRIAMBOAVONJY, V., ROUL, G., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1999). Mechanism of Ca2+ release and entry during contraction elicited by norepinephrine in rat resistance arteries. *Am J Physiol*, **276,** H300-8.

LAMBERT, G., SCHLAICH, M., LAMBERT, E., DAWOOD, T. & ESLER, M. (2010). Stress reactivity and its association with increased cardiovascular risk: a role for the sympathetic nervous system? *Hypertension*, **55,** e20; author reply e21.

LAMONT, C., VAINORIUS, E. & WIER, W.G. (2003). Purinergic and adrenergic Ca2+ transients during neurogenic contractions of rat mesenteric small arteries. *J Physiol*, **549,** 801-8.

LAMONT, C. & WIER, W.G. (2004). Different roles of ryanodine receptors and inositol (1,4,5)-trisphosphate receptors in adrenergically stimulated contractions of small arteries. *Am J Physiol Heart Circ Physiol*, **287,** H617-25.

LARGE, W.A., SALEH, S.N. & ALBERT, A.P. (2009). Role of phosphoinositol 4,5-bisphosphate and diacylglycerol in regulating native TRPC channel proteins in vascular smooth muscle. *Cell Calcium*, **45,** 574-82.

LEBLANC, N., LEDOUX, J., SALEH, S., SANGUINETTI, A., ANGERMANN, J., O'DRISCOLL, K., BRITTON, F., PERRINO, B.A. & GREENWOOD, I.A. (2005). Regulation of calcium-activated chloride channels in smooth muscle cells: a complex picture is emerging. *Can J Physiol Pharmacol*, **83,** 541-56.

LEDOUX, J., TAYLOR, M.S., BONEV, A.D., HANNAH, R.M., SOLODUSHKO, V., SHUI, B., TALLINI, Y., KOTLIKOFF, M.I. & NELSON, M.T. (2008). Functional architecture of inositol 1,4,5-trisphosphate signaling in restricted spaces of myoendothelial projections. *Proc Natl Acad Sci U S A*, **105,** 9627-32.

LEUNG, Y.K., DU, J., HUANG, Y. & YAO, X. (2010). Cyclic nucleotide-gated channels contribute to thromboxane A2-induced contraction of rat small mesenteric arteries. *PLoS One*, **5,** e11098.

LEURANGUER, V., VANHOUTTE, P.M., VERBEUREN, T. & FELETOU, M. (2008). C-type natriuretic peptide and endothelium-dependent hyperpolarization in the guinea-pig carotid artery. *Br J Pharmacol*, **153,** 57-65.

LEVY, M.N. (1979). The cardiac and vascular factors that determine systemic blood flow. *Circ Res*, **44,** 739-47.

LEW, M.J., RIVERS, R.J. & DULING, B.R. (1989). Arteriolar smooth muscle responses are modulated by an intramural diffusion barrier. *Am J Physiol*, **257,** H10-6.

LI, C.G., KARAGIANNIS, J. & RAND, M.J. (1999a). Comparison of the redox forms of nitrogen monoxide with the nitrergic transmitter in the rat anococcygeus muscle. *Br J Pharmacol*, **127,** 826-34.

LI, C.G. & RAND, M.J. (1999b). Effects of hydroxocobalamin and carboxy-PTIO on nitrergic transmission in porcine anococcygeus and retractor penis muscles. *Br J Pharmacol*, **127,** 172-6.

LI, H., WITTE, K., AUGUST, M., BRAUSCH, I., GODTEL-ARMBRUST, U., HABERMEIER, A., CLOSS, E.I., OELZE, M., MUNZEL, T. & FORSTERMANN, U. (2006). Reversal of endothelial nitric oxide synthase uncoupling and up-regulation of endothelial nitric oxide synthase expression lowers blood pressure in hypertensive rats. *J Am Coll Cardiol*, **47,** 2536-44.

LI, J., ZHOU, Z., JIANG, D.J., LI, D., TAN, B., LIU, H. & LI, Y.J. (2007). Reduction of NO- and EDHF-mediated vasodilatation in hypertension: role of asymmetric dimethylarginine. *Clin Exp Hypertens*, **29,** 489-501.

LIGGETT, S.B., FREEDMAN, N.J., SCHWINN, D.A. & LEFKOWITZ, R.J. (1993). Structural basis for receptor subtype-specific regulation revealed by a chimeric beta 3/beta 2-adrenergic receptor. *Proc Natl Acad Sci U S A*, **90,** 3665-9.

LINDER, L., KIOWSKI, W., BUHLER, F.R. & LUSCHER, T.F. (1990). Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. *Circulation*, **81,** 1762-7.

LIU, C., NGAI, C.Y., HUANG, Y., KO, W.H., WU, M., HE, G.W., GARLAND, C.J., DORA, K.A. & YAO, X. (2006). Depletion of intracellular Ca2+ stores enhances flow-induced vascular dilatation in rat small mesenteric artery. *Br J Pharmacol*, **147,** 506-15.

LIU, C.Q., LEUNG, F.P., WONG, S.L., WONG, W.T., LAU, C.W., LU, L., YAO, X., YAO, T. & HUANG, Y. (2009). Thromboxane prostanoid receptor activation impairs endothelial nitric oxide-dependent vasorelaxations: the role of Rho kinase. *Biochem Pharmacol*, **78,** 374-81.

LIU, C.Q., WONG, S.L., LEUNG, F.P., TIAN, X.Y., LAU, C.W., LU, L., YAO, X., CHEN, Z.Y., YAO, T. & HUANG, Y. (2010). Prostanoid TP receptor-mediated impairment of cyclic AMP-dependent vasorelaxation is reversed by phosphodiesterase inhibitors. *Eur J Pharmacol*, **632,** 45-51.

LIU, H., XIONG, Z. & SPERELAKIS, N. (1997). Cyclic nucleotides regulate the activity of L-type calcium channels in smooth muscle cells from rat portal vein. *J Mol Cell Cardiol*, **29,** 1411-21.

LIU, M., LARGE, W.A. & ALBERT, A.P. (2005). Stimulation of beta-adrenoceptors inhibits store-operated channel currents via a cAMP-dependent protein kinase mechanism in rabbit portal vein myocytes. *J Physiol*, **562,** 395-406.

LUCCHESI, P.A., BELMADANI, S. & MATROUGUI, K. (2005). Hydrogen peroxide acts as both vasodilator and vasoconstrictor in the control of perfused mouse mesenteric resistance arteries. *J Hypertens*, **23,** 571-9.

LUCKHOFF, A., MULSCH, A. & BUSSE, R. (1990). cAMP attenuates autacoid release from endothelial cells: relation to internal calcium. *Am J Physiol*, **258,** H960-6.

MACDONALD, A., MCLEAN, M., MACAULAY, L. & SHAW, A.M. (1999). Effects of propranolol and L-NAME on beta-adrenoceptor-mediated relaxation in rat carotid artery. *J Auton Pharmacol*, **19,** 145-9.

MACDONALD, P.S., DUBBIN, P.N. & DUSTING, G.J. (1987). Beta-adrenoceptors on endothelial cells do not influence release of relaxing factor in dog coronary arteries. *Clin Exp Pharmacol Physiol*, **14,** 525-34.

MAGUIRE, J.J., KUC, R.E., O'REILLY, G. & DAVENPORT, A.P. (1994). Vasoconstrictor endothelin receptors characterized in human renal artery and vein in vitro. *Br J Pharmacol*, **113,** 49-54.

MAJEWSKI, H., TUNG, L.H. & RAND, M.J. (1981). Adrenaline-induced hypertension in rats. *J Cardiovasc Pharmacol*, **3,** 179-85.

MANARA, L., BADONE, D., BARONI, M., BOCCARDI, G., CECCHI, R., CROCI, T., GIUDICE, A., GUZZI, U., LANDI, M. & LE FUR, G. (1996). Functional identification of rat atypical beta-adrenoceptors by the first beta 3-selective antagonists, aryloxypropanolaminotetralins. *Br J Pharmacol*, **117,** 435-442.

MANIATIS, N.A., BROVKOVYCH, V., ALLEN, S.E., JOHN, T.A., SHAJAHAN, A.N., TIRUPPATHI, C., VOGEL, S.M., SKIDGEL, R.A., MALIK, A.B. & MINSHALL, R.D. (2006). Novel mechanism of endothelial nitric oxide synthase activation mediated by caveolae internalization in endothelial cells. *Circ Res*, **99,** 870-7.

MARCANTONI, A., BALDELLI, P., HERNANDEZ-GUIJO, J.M., COMUNANZA, V., CARABELLI, V. & CARBONE, E. (2007). L-type calcium channels in adrenal chromaffin cells: role in pace-making and secretion. *Cell Calcium*, **42,** 397-408.

MARCHENKO, S.M. & SAGE, S.O. (1994). Smooth muscle cells affect endothelial membrane potential in rat aorta. *Am J Physiol*, **267,** H804-11.

MARTIN, W. (2009). Nitroxyl anion--the universal signalling partner of endogenously produced nitric oxide? *Br J Pharmacol*, **157,** 537-9.

MARTIN, W., FURCHGOTT, R.F., VILLANI, G.M. & JOTHIANANDAN, D. (1986). Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J Pharmacol Exp Ther*, **237,** 529-38.

MATCHKOV, V.V. (2010). Mechanisms of cellular synchronization in the vascular wall. Mechanisms of vasomotion. *Dan Med Bull*, **57,** B4191.

MATHER, S., DORA, K.A., SANDOW, S.L., WINTER, P. & GARLAND, C.J. (2005). Rapid endothelial cell-selective loading of connexin 40 antibody blocks endothelium-derived hyperpolarizing factor dilation in rat small mesenteric arteries. *Circ Res*, **97,** 399-407.

MATOBA, T. & SHIMOKAWA, H. (2003). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. *J Pharmacol Sci*, **92,** 1-6.

MATSUBARA, M., HAYASHI, N., JING, T. & TITANI, K. (2003). Regulation of endothelial nitric oxide synthase by protein kinase C. *J Biochem*, **133,** 773-81.

MATSUDA, N., HAYASHI, Y., TAKAHASHI, Y. & HATTORI, Y. (2006). Phosphorylation of endothelial nitric-oxide synthase is diminished in mesenteric arteries from septic rabbits depending on the altered phosphatidylinositol 3-kinase/Akt pathway: reversal effect of fluvastatin therapy. *J Pharmacol Exp Ther*, **319,** 1348-54.

MAUBAN, J.R. & WIER, W.G. (2004). Essential role of EDHF in the initiation and maintenance of adrenergic vasomotion in rat mesenteric arteries. *Am J Physiol Heart Circ Physiol*, **287,** H608-16.

MCKENZIE, C., MACDONALD, A. & SHAW, A.M. (2009). Mechanisms of U46619-induced contraction of rat pulmonary arteries in the presence and absence of the endothelium. *Br J Pharmacol*, **157,** 581-96.

MCNEISH, A.J. & GARLAND, C.J. (2007). Thromboxane A2 inhibition of SKCa after NO synthase block in rat middle cerebral artery. *Br J Pharmacol*, **151,** 441-9.

MCSHERRY, I.N., SPITALER, M.M., TAKANO, H. & DORA, K.A. (2005). Endothelial cell Ca2+ increases are independent of membrane potential in pressurized rat mesenteric arteries. *Cell Calcium*, **38,** 23-33.

MECHOULAM, R., FRIDE, E., BEN-SHABAT, S., MEIRI, U. & HOROWITZ, M. (1998). Carbachol, an acetylcholine receptor agonist, enhances production in rat aorta of 2-arachidonoyl glycerol, a hypotensive endocannabinoid. *Eur J Pharmacol*, **362,** R1-3.

MEHTA, D. & MALIK, A.B. (2006). Signaling mechanisms regulating endothelial permeability. *Physiol Rev*, **86,** 279-367.

MILLER, O.V., AIKEN, J.W., HEMKER, D.P., SHEBUSKI, R.J. & GORMAN, R.R. (1979). Prostacyclin stimulation of dog arterial cyclic AMP levels. *Prostaglandins*, **18,** 915-25.

MING, X.F., VISWAMBHARAN, H., BARANDIER, C., RUFFIEUX, J., KAIBUCHI, K., RUSCONI, S. & YANG, Z. (2002). Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Mol Cell Biol*, **22,** 8467-77.

MISTRY, D.K. & GARLAND, C.J. (1998). Nitric oxide (NO)-induced activation of large conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) in smooth muscle cells isolated from the rat mesenteric artery. *Br. J. Pharmacol.*, **124,** 1131-1148.

MODIN, A., BJORNE, H., HERULF, M., ALVING, K., WEITZBERG, E. & LUNDBERG, J.O. (2001). Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand*, **171,** 9-16.

MONCADA, S., GRYGLEWSKI, R., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263,** 663-5.

MONCADA, S. & HIGGS, E.A. (2006). The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol*, **147 Suppl 1,** S193-201.

MONTAGNANI, M., VULPIS, V., NAZZARO, P., POTENZA, M.A., RINALDI, R., NACCI, C., DE SALVIA, M.A., SIRO BRIGIANI, G., PIRRELLI, A. & MITOLO-CHIEPPA, D. (2000). Endothelin-1-receptor-mediated responses in resistance vessels of young and adult spontaneously hypertensive rats. *J Hypertens*, **18,** 893-900.

MONTI, M., DONNINI, S., GIACHETTI, A., MOCHLY-ROSEN, D. & ZICHE, M. (2010). deltaPKC inhibition or varepsilonPKC activation repairs endothelial vascular dysfunction by regulating eNOS post-translational modification. *J Mol Cell Cardiol*, **48,** 746-56.

MOOSMANG, S., SCHULLA, V., WELLING, A., FEIL, R., FEIL, S., WEGENER, J.W., HOFMANN, F. & KLUGBAUER, N. (2003). Dominant role of smooth muscle L-type calcium channel Cav1.2 for blood pressure regulation. *Embo J*, **22,** 6027-34.

MULVANY, M.J. & AALKJAER, C. (1990). Structure and function of small arteries. *Physiol Rev*, **70,** 921-61.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res*, **41,** 19-26.

MULVANY, M.J., NILSSON, H. & FLATMAN, J.A. (1982). Role of membrane potential in the response of rat small mesenteric arteries to exogenous noradrenaline stimulation. *J Physiol*, **332,** 363-73.

MUMTAZ, S., BURDYGA, G., BORISOVA, L., WRAY, S. & BURDYGA, T. (2010). The mechanism of agonist induced Ca(2+) signalling in intact endothelial cells studied confocally in in situ arteries. *Cell Calcium*.

MUMTAZ, S., BURDYGA, G., BORISOVA, L., WRAY, S. & BURDYGA, T. (2011). The mechanism of agonist induced Ca(2+) signalling in intact endothelial cells studied confocally in in situ arteries. *Cell Calcium*.

MURAKI, K., WATANABE, M. & IMAIZUMI, Y. (2000). Nifedipine and nisoldipine modulate membrane potential of vascular endothelium via a myo-endothelial pathway. *Life Sci*, **67,** 3163-70.

NAGASE, S., TAKEMURA, K., UEDA, A., HIRAYAMA, A., AOYAGI, K., KONDOH, M. & KOYAMA, A. (1997). A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D- or L-arginine. *Biochem Biophys Res Commun*, **233,** 150-3.

NAKASHIMA, M. & VANHOUTTE, P.M. (1995). Isoproterenol causes hyperpolarization through opening of ATP-sensitive potassium channels in vascular smooth muscle of the canine saphenous vein. *J Pharmacol Exp Ther*, **272,** 379-84.

NAPP, A., BRIXIUS, K., POTT, C., ZISKOVEN, C., BOELCK, B., MEHLHORN, U., SCHWINGER, R.H. & BLOCH, W. (2009). Effects of the beta3-adrenergic agonist BRL 37344 on endothelial nitric oxide synthase phosphorylation and force of contraction in human failing myocardium. *J Card Fail*, **15,** 57-67.

NELSON, M.T. & QUAYLE, J.M. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol*, **268,** C799-822.

NELSON, M.T., STANDEN, N.B., BRAYDEN, J.E. & WORLEY, J.F., 3RD (1988). Noradrenaline contracts arteries by activating voltage-dependent calcium channels. *Nature*, **336,** 382-5.

NEUMAN, I., MALOBERTI, P., LISDERO, C., COLONNA, C., PERALTA, J., JOSE, J.P. & PODESTA, E.J. (2002). beta-Adrenergic stimulation controls the expression of a thioesterase specific for very-long-chain fatty acids in perfused hearts. *Biochem Biophys Res Commun*, **299,** 135-41.

NEYLON, C.B., D'SOUZA, T. & REINHART, P.H. (2004). Protein kinase A inhibits intermediate conductance Ca2+-activated K+ channels expressed in Xenopus oocytes. *Pflugers Arch*, **448,** 613-20.

NICHOLSON, N.S., SMITH, S.L. & FULLER, G.C. (1984). Effect of the stable endoperoxide analog U-46619 on prostacyclin production and cyclic AMP levels in bovine endothelial cells. *Thromb Res*, **35,** 183-92.

NILIUS, B. (2004). Store-operated Ca2+ entry channels: still elusive! *Sci STKE*, **2004,** pe36.

NILSSON, H., VIDEBAEK, L.M., TOMA, C. & MULVANY, M.J. (1998). Role of intracellular calcium for noradrenaline-induced depolarization in rat mesenteric small arteries. *J Vasc Res*, **35,** 36-44.

NISHINA, H., OZAKI, T., HANSON, M.A. & POSTON, L. (1999). Mechanisms of noradrenaline-induced vasorelaxation in isolated femoral arteries of the neonatal rat. *Br J Pharmacol*, **127,** 809-12.

OISHI, H., BUDEL, S., SCHUSTER, A., STERGIOPULOS, N., MEISTER, J.J. & BENY, J.L. (2001). Cytosolic-free calcium in smooth-muscle and endothelial cells in an intact arterial wall from rat mesenteric artery in vitro. *Cell Calcium*, **30,** 261-7.

OLIVER, G. & SCHAFER, E.A. (1895). The Physiological Effects of Extracts of the Suprarenal Capsules. *J Physiol*, **18,** 230-76.

OMAR, R., BOTTRILL, F.E., HILEY, C.R. & WHITE, R. (2000). Interaction of cyclic AMP modulating agents with levcromakalim in the relaxation of rat isolated mesenteric artery. *Eur J Pharmacol*, **401,** 85-96.

ORLOV, S.N., TREMBLAY, J. & HAMET, P. (1996). cAMP signaling inhibits dihydropyridine-sensitive Ca2+ influx in vascular smooth muscle cells. *Hypertension*, **27,** 774-80.

PAIVA, M.Q., MORATO, M., MOURA, D. & GUIMARAES, S. (1999). A comparative study of postsynaptic alpha2-adrenoceptors of the dog mesenteric and rat femoral veins. *Naunyn Schmiedebergs Arch Pharmacol*, **360,** 165-70.

PALMER, R.M., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333,** 664-6.

PALMER, R.M., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327,** 524-6.

PALTAUF-DOBURZYNSKA, J., FRIEDEN, M., SPITALER, M. & GRAIER, W.F. (2000). Histamine-induced Ca2+ oscillations in a human endothelial cell line depend on transmembrane ion flux, ryanodine receptors and endoplasmic reticulum Ca2+-ATPase. *J Physiol*, **524 Pt 3,** 701-13.

PENG, H., MATCHKOV, V., IVARSEN, A., AALKJAER, C. & NILSSON, H. (2001). Hypothesis for the initiation of vasomotion. *Circ Res*, **88,** 810-5.

PEREZ-VIZCAINO, F., VILLAMOR, E., DUARTE, J. & TAMARGO, J. (1997). Involvement of protein kinase C in reduced relaxant responses to the NO/cyclic GMP pathway in piglet pulmonary arteries contracted by the thromboxane A2-mimetic U46619. *Br J Pharmacol*, **121,** 1323-33.

PETKOV, G.V. & NELSON, M.T. (2005). Differential regulation of Ca2+-activated K+ channels by beta-adrenoceptors in guinea pig urinary bladder smooth muscle. *Am J Physiol Cell Physiol*, **288,** C1255-63.

PFISTER, S.L. (2008). Characterization of endothelial thromboxane receptors in rabbit aorta. *Prostaglandins Other Lipid Mediat*, **87,** 54-61.

PIASCIK, M.T., HROMETZ, S.L., EDELMANN, S.E., GUARINO, R.D., HADLEY, R.W. & BROWN, R.D. (1997). Immunocytochemical localization of the alpha-1B adrenergic receptor and the contribution of this and the other subtypes to vascular smooth muscle contraction: analysis with selective ligands and antisense oligonucleotides. *J Pharmacol Exp Ther*, **283,** 854-68.

PIMENTEL, A.M., COSTA, C.A., CARVALHO, L.C., BRANDAO, R.M., RANGEL, B.M., TANO, T., SOARES DE MOURA, R. & RESENDE, A.C. (2007). The role of NO-cGMP pathway and potassium channels on the relaxation induced by clonidine in the rat mesenteric arterial bed. *Vascul Pharmacol*, **46,** 353-9.

PINO, R.Z. & FEELISCH, M. (1994a). Bioassay discrimination between nitric oxide (NO.) and nitroxyl (NO-) using L-cysteine. *Biochem Biophys Res Commun*, **201,** 54-62.

PINO, R.Z. & FEELISCH, M. (1994b). Bioassay discrimination between nitric oxide ($NO^{\bullet}$) and nitroxyl (NO^{-}) using L-cysteine. *Biochem Biophys Res Commun*, **201,** 54-62.

PINTEROVA, M., KAREN, P., KUNES, J. & ZICHA, J. (2010). Role of nifedipine-sensitive sympathetic vasoconstriction in maintenance of high blood pressure in spontaneously hypertensive rats: effect of Gi-protein inactivation by pertussis toxin. *J Hypertens*, **28,** 969-78.

PIPER, A.S. & LARGE, W.A. (2004). Direct effect of Ca2+-calmodulin on cGMP-activated Ca2+-dependent Cl-channels in rat mesenteric artery myocytes. *J Physiol*, **559,** 449-57.

PLANE, F. & GARLAND, C.J. (1996). Influence of contractile agonists on the mechanism of endothelium-dependent relaxation in rat isolated mesenteric artery. *Br J Pharmacol*, **119,** 191-3.

POHL, J., WINDER, S.J., ALLEN, B.G., WALSH, M.P., SELLERS, J.R. & GERTHOFFER, W.T. (1997). Phosphorylation of calponin in airway smooth muscle. *Am J Physiol*, **272,** L115-23.

PONSIOEN, B., VAN ZEIJL, L., MOOLENAAR, W.H. & JALINK, K. (2007). Direct measurement of cyclic AMP diffusion and signaling through connexin43 gap junctional channels. *Exp Cell Res*, **313,** 415-23.

POPP, R., BRANDES, R.P., OTT, G., BUSSE, R. & FLEMING, I. (2002). Dynamic modulation of interendothelial gap junctional communication by 11,12-epoxyeicosatrienoic acid. *Circ Res*, **90,** 800-6.

PRADIDARCHEEP, W., STALLEN, J., LABRUYERE, W.T., DABHOIWALA, N.F., MICHEL, M.C. & LAMERS, W.H. (2009). Lack of specificity of commercially available antisera against muscarinergic and adrenergic receptors. *Naunyn Schmiedebergs Arch Pharmacol*, **379,** 397-402.

PREHN, J.L. & BEVAN, J.A. (1983). Facial vein of the rabbit. Intracellularly recorded hyperpolarization of smooth muscle cells induced by beta-adrenergic receptor stimulation. *Circ Res*, **52,** 465-70.

PRIVIERO, F.B., TEIXEIRA, C.E., CLAUDINO, M.A., DE NUCCI, G., ZANESCO, A. & ANTUNES, E. (2007). Vascular effects of long-term propranolol administration after chronic nitric oxide blockade. In *Eur J Pharmacol*. pp. 189-96.

PURVES, G.I., KAMISHIMA, T., DAVIES, L.M., QUAYLE, J.M. & DART, C. (2009). Exchange protein activated by cAMP (Epac) mediates cAMP-dependent but protein kinase A-insensitive modulation of vascular ATP-sensitive potassium channels. *J Physiol*, **587,** 3639-50.

QUAYLE, J.M., NELSON, M.T. & STANDEN, N.B. (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol Rev*, **77,** 1165-232.

QUINN, K.V., GIBLIN, J.P. & TINKER, A. (2004). Multisite phosphorylation mechanism for protein kinase A activation of the smooth muscle ATP-sensitive K+ channel. *Circ Res*, **94,** 1359-66.

RAHMAN, A., HUGHES, A., MATCHKOV, V., NILSSON, H. & AALKJAER, C. (2007). Antiphase oscillations of endothelium and smooth muscle [Ca2+]i in vasomotion of rat mesenteric small arteries. *Cell Calcium*, **42,** 536-47.

RAHMAN, A., MATCHKOV, V., NILSSON, H. & AALKJAER, C. (2005). Effects of cGMP on coordination of vascular smooth muscle cells of rat mesenteric small arteries. *J Vasc Res*, **42,** 301-11.

RAINBOW, R.D., NORMAN, R.I., EVERITT, D.E., BRIGNELL, J.L., DAVIES, N.W. & STANDEN, N.B. (2009). Endothelin-I and angiotensin II inhibit arterial voltage-gated K+ channels through different protein kinase C isoenzymes. *Cardiovasc Res*, **83,** 493-500.

RAJANAYAGAM, M.A., LI, C.G. & RAND, M.J. (1993). Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle. *Br J Pharmacol*, **108,** 3-5.

RANDALL, M.D., MCCULLOCH, A.I. & KENDALL, D.A. (1997). Comparative pharmacology of endothelium-derived hyperpolarizing factor and anandamide in rat isolated mesentery. *Eur J Pharmacol*, **333,** 191-7.

RASCADO, R.R. & BENDHACK, L.M. (2005). Activation of alpha2-adrenoceptors is necessary to induce nitric oxide release in isoprenaline-induced relaxation. *Vascul Pharmacol*, **42,** 63-8.

RATH, G., DESSY, C. & FERON, O. (2009). Caveolae, caveolin and control of vascular tone: nitric oxide (NO) and endothelium derived hyperpolarizing factor (EDHF) regulation. *J Physiol Pharmacol*, **60 Suppl 4,** 105-9.

RATZ, P.H., BERG, K.M., URBAN, N.H. & MINER, A.S. (2005). Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *Am J Physiol Cell Physiol*, **288,** C769-83.

RAYCHOWDHURY, M.K., YUKAWA, M., COLLINS, L.J., MCGRAIL, S.H., KENT, K.C. & WARE, J.A. (1994). Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A2 receptor. *J Biol Chem*, **269,** 19256-61.

REITER, M.J. (2004). Cardiovascular drug class specificity: beta-blockers. *Prog Cardiovasc Dis*, **47,** 11-33.

RHODIN, J.A. (1967). The ultrastructure of mammalian arterioles and precapillary sphincters. *J Ultrastruct Res*, **18,** 181-223.

RODRIGUEZ-RODRIGUEZ, R., YAROVA, P., WINTER, P. & DORA, K.A. (2009). Desensitization of endothelial P2Y1 receptors by PKC-dependent mechanisms in pressurized rat small mesenteric arteries. *Br J Pharmacol*, **158,** 1609-20.

SAMOKOVLISKY, A., RIMON, G. & DANON, A. (1999). Differential regulation of cyclooxygenase isoenzymes by cAMP-elevating agents. *Eur J Pharmacol*, **378,** 203-11.

SAMPSON, L.J., DAVIES, L.M., BARRETT-JOLLEY, R., STANDEN, N.B. & DART, C. (2007). Angiotensin II-activated protein kinase C targets caveolae to inhibit aortic ATP-sensitive potassium channels. *Cardiovasc Res*, **76,** 61-70.

SAMPSON, L.J., PLANE, F. & GARLAND, C.J. (2001). Involvement of cyclic GMP and potassium channels in relaxation evoked by the nitric oxide donor, diethylamine NONOate, in the rat small isolated mesenteric artery. *Naunyn Schmiedebergs Arch Pharmacol*, **364,** 220-5.

SANDOW, S.L., BRAMICH, N.J., BANDI, H.P., RUMMERY, N.M. & HILL, C.E. (2003). Structure, function, and endothelium-derived hyperpolarizing factor in the caudal artery of the SHR and WKY rat. *Arterioscler Thromb Vasc Biol*, **23,** 822-8.

SANDOW, S.L., GOTO, K., RUMMERY, N.M. & HILL, C.E. (2004). Developmental changes in myoendothelial gap junction mediated vasodilator activity in the rat saphenous artery. *J Physiol*, **556,** 875-86.

SANDOW, S.L., HADDOCK, R.E., HILL, C.E., CHADHA, P.S., KERR, P.M., WELSH, D.G. & PLANE, F. (2009). What's where and why at a vascular myoendothelial microdomain signalling complex. *Clin Exp Pharmacol Physiol*, **36,** 67-76.

SANDOW, S.L. & HILL, C.E. (2000). Incidence of myoendothelial gap junctions in the proximal and distal mesenteric arteries of the rat is suggestive of a role in endothelium-derived hyperpolarizing factor-mediated responses. *Circ Res*, **86,** 341-6.

SANDOW, S.L., NEYLON, C.B., CHEN, M.X. & GARLAND, C.J. (2006). Spatial separation of endothelial small- and intermediate-conductance calcium-activated potassium channels (K(Ca)) and connexins: possible relationship to vasodilator function? *J Anat*, **209,** 689-98.

SANDOW, S.L., TARE, M., COLEMAN, H.A., HILL, C.E. & PARKINGTON, H.C. (2002). Involvement of myoendothelial gap junctions in the actions of endothelium-derived hyperpolarizing factor. *Circ Res*, **90,** 1108-13.

SCHMIDT, H.H., HOFMANN, H., SCHINDLER, U., SHUTENKO, Z.S., CUNNINGHAM, D.D. & FEELISCH, M. (1996). No .NO from NO synthase. *Proc Natl Acad Sci U S A*, **93,** 14492-7.

SCHMIDT, M., EVELLIN, S., WEERNINK, P.A., VON DORP, F., REHMANN, H., LOMASNEY, J.W. & JAKOBS, K.H. (2001). A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nat Cell Biol*, **3,** 1020-4.

SCHNEIDER, J.C., EL KEBIR, D., CHEREAU, C., MERCIER, J.C., DALL'AVA-SANTUCCI, J. & DINH-XUAN, A.T. (2002). Involvement of Na(+)/Ca(2+) exchanger in endothelial NO production and endothelium-dependent relaxation. *Am J Physiol Heart Circ Physiol*, **283,** H837-44.

SCHULZ, R., SCHMIDT, D., BLUM, A., LOPES-RIBEIRO, X., LUCKE, C., MAYER, K., OLSCHEWSKI, H., SEEGER, W. & GRIMMINGER, F. (2000). Decreased plasma levels of nitric oxide derivatives in obstructive sleep apnoea: response to CPAP therapy. *Thorax*, **55,** 1046-51.

SEGAL, S.S. & DULING, B.R. (1989). Conduction of vasomotor responses in arterioles: a role for cell-to-cell coupling? *Am J Physiol*, **256,** H838-45.

SEGAL, S.S., WELSH, D.G. & KURJIAKA, D.T. (1999). Spread of vasodilatation and vasoconstriction along feed arteries and arterioles of hamster skeletal muscle. *J Physiol*, **516 (Pt 1),** 283-91.

SELL, M., BOLDT, W. & MARKWARDT, F. (2002). Desynchronising effect of the endothelium on intracellular Ca2+ concentration dynamics in vascular smooth muscle cells of rat mesenteric arteries. *Cell Calcium*, **32,** 105-20.

SEYA, Y., FUKUDA, T., ISOBE, K., KAWAKAMI, Y. & TAKEKOSHI, K. (2006). Effect of norepinephrine on RhoA, MAP kinase, proliferation and VEGF expression in human umbilical vein endothelial cells. *Eur J Pharmacol*, **553,** 54-60.

SHARPE, M.A. & COOPER, C.E. (1998). Reactions of nitric oxide with mitochondrial cytochrome c: a novel mechanism for the formation of nitroxyl anion and peroxynitrite. *Biochem J*, **332 (Pt 1),** 9-19.

SHAW, L., O'NEILL, S., JONES, C.J., AUSTIN, C. & TAGGART, M.J. (2004). Comparison of U46619-, endothelin-1- or phenylephrine-induced changes in cellular Ca2+ profiles and Ca2+ sensitisation of constriction of pressurised rat resistance arteries. *Br J Pharmacol*, **141,** 678-88.

SHEN, B., CHENG, K.T., LEUNG, Y.K., KWOK, Y.C., KWAN, H.Y., WONG, C.O., CHEN, Z.Y., HUANG, Y. & YAO, X. (2008). Epinephrine-induced Ca2+ influx in vascular endothelial cells is mediated by CNGA2 channels. *J Mol Cell Cardiol*, **45,** 437-45.

SHEN, Y.T., ZHANG, H. & VATNER, S.F. (1994). Peripheral vascular effects of beta-3 adrenergic receptor stimulation in conscious dogs. *J Pharmacol Exp Ther*, **268,** 466-73.

SHI, Y., WU, Z., CUI, N., SHI, W., YANG, Y., ZHANG, X., ROJAS, A., HA, B.T. & JIANG, C. (2007). PKA phosphorylation of SUR2B subunit underscores vascular KATP channel activation by beta-adrenergic receptors. *Am J Physiol Regul Integr Comp Physiol*, **293,** R1205-14.

SHIBASAKI, T. (1987). Conductance and kinetics of delayed rectifier potassium channels in nodal cells of the rabbit heart. *J Physiol*, **387,** 227-50.

SHIMOKAWA, H. & MATOBA, T. (2004). Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pharmacol Res*, **49,** 543-9.

SHIRASAKI, H., KIKUCHI, M., SEKI, N., KANAIZUMI, E., WATANABE, K. & HIMI, T. (2007). Expression and localization of the thromboxane A2 receptor in human nasal mucosa. *Prostaglandins Leukot Essent Fatty Acids*, **76,** 315-20.

SILVA, E.G., FERES, T., VIANNA, L.M., OKUYAMA, P. & PAIVA, T.B. (1996). Dual effect of clonidine on mesenteric artery adrenoceptors: agonistic (alpha-2) and antagonistic (alpha-1). *J Pharmacol Exp Ther*, **277,** 872-6.

SIMONDS, W.F. (1999). G protein regulation of adenylate cyclase. *Trends Pharmacol Sci*, **20,** 66-73.

SIMONSEN, U., WADSWORTH, R.M., BUUS, N.H. & MULVANY, M.J. (1999). In vitro simultaneous measurements of relaxation and nitric oxide concentration in rat superior mesenteric artery. *J Physiol*, **516 (Pt 1),** 271-82.

SMIRNOV, S.V. & AARONSON, P.I. (1992). Ca2+ currents in single myocytes from human mesenteric arteries: evidence for a physiological role of L-type channels. *J Physiol*, **457,** 455-75.

SOBEY, C.G., HEISTAD, D.D. & FARACI, F.M. (1998). Potassium channels mediate dilatation of cerebral arterioles in response to arachidonate. *Am J Physiol*, **275,** H1606-12.

SOMASUNDARAM, C., DIZ, D.I., COLEMAN, T. & BUKOSKI, R.D. (2006). Adventitial neuronal somata. *J Vasc Res*, **43,** 278-88.

SOMLYO, A.P. & SOMLYO, A.V. (2003). Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev*, **83,** 1325-58.

SOMLYO, A.V., HAEUSLER, G. & SOMLYO, A.P. (1970). Cyclic adenosine monophosphate: potassium-dependent action on vascular smooth muscle membrane potential. *Science*, **169,** 490-1.

SONG, P., ZHANG, M., WANG, S., XU, J., CHOI, H.C. & ZOU, M.H. (2009). Thromboxane A2 receptor activates a Rho-associated kinase/LKB1/PTEN pathway to attenuate endothelium insulin signaling. *J Biol Chem*, **284,** 17120-8.

SOOCH, S. & MARSHALL, I. (1995). An atypical β-adrenoceptor mediates relaxation of the rat isolated mesenteric artery. *Br. J. Pharmacol.*, **114:22P**.

STEINERT, D., KUPER, C., BARTELS, H., BECK, F.X. & NEUHOFER, W. (2009). PGE2 potentiates tonicity-induced COX-2 expression in renal medullary cells in a positive feedback loop involving EP2-cAMP-PKA signaling. *Am J Physiol Cell Physiol*, **296,** C75-87.

SUGIMOTO, K.I., SHIGA, T. & FUJIMURA, A. (2000). Delayed hypotensive effect of the thromboxane A2/prostaglandin H2 receptor antagonist S-1452 in spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol*, **27,** 594-600.

SUMII, K. & SPERELAKIS, N. (1995). cGMP-dependent protein kinase regulation of the L-type Ca2+ current in rat ventricular myocytes. *Circ Res*, **77,** 803-12.

TAKANO, H., DORA, K.A., SPITALER, M.M. & GARLAND, C.J. (2004). Spreading dilatation in rat mesenteric arteries associated with calcium-independent endothelial cell hyperpolarization. *J Physiol*, **556,** 887-903.

TALLINI, Y.N., BREKKE, J.F., SHUI, B., DORAN, R., HWANG, S.M., NAKAI, J., SALAMA, G., SEGAL, S.S. & KOTLIKOFF, M.I. (2007). Propagated endothelial Ca2+ waves and arteriolar dilation in vivo: measurements in Cx40BAC GCaMP2 transgenic mice. *Circ Res*, **101,** 1300-9.

TAMMARO, P., SMITH, A.L., CROWLEY, B.L. & SMIRNOV, S.V. (2005). Modulation of the voltage-dependent K+ current by intracellular Mg2+ in rat aortic smooth muscle cells. *Cardiovasc Res*, **65,** 387-96.

TANG, E.H., LEUNG, F.P., HUANG, Y., FELETOU, M., SO, K.F., MAN, R.Y. & VANHOUTTE, P.M. (2007). Calcium and reactive oxygen species increase in endothelial cells in response to releasers of endothelium-derived contracting factor. *Br J Pharmacol*, **151,** 15-23.

TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol Sci*, **9,** 272-4.

TELEZHKIN, V.S., YAROVA, P.L., DORA, K.A. & GARLAND, C.J. (unpublished). Novel Characteristics of Native Endothelial Cells from Rat Mesenteric Resistance Arteries.

TIAN, L., COGHILL, L.S., MCCLAFFERTY, H., MACDONALD, S.H., ANTONI, F.A., RUTH, P., KNAUS, H.G. & SHIPSTON, M.J. (2004). Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A*, **101,** 11897-902.

TIRUPPATHI, C., AHMMED, G.U., VOGEL, S.M. & MALIK, A.B. (2006). Ca2+ signaling, TRP channels, and endothelial permeability. *Microcirculation*, **13,** 693-708.

TOCCHETTI, C.G., WANG, W., FROEHLICH, J.P., HUKE, S., AON, M.A., WILSON, G.M., DI BENEDETTO, G., O'ROURKE, B., GAO, W.D., WINK, D.A., TOSCANO, J.P., ZACCOLO, M., BERS, D.M., VALDIVIA, H.H., CHENG, H., KASS, D.A. & PAOLOCCI, N. (2007). Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca2+ cycling. *Circ Res*, **100,** 96-104.

TORRENS, C., BRAWLEY, L., ITOH, S., POSTON, L. & HANSON, M.A. (2002). Atypical beta-adrenoceptor-mediated vasodilatation in rat isolated small mesenteric arteries. *Br J Pharmacol*, **135**.

TOYOSHIMA, H., NASA, Y., HASHIZUME, Y., KOSEKI, Y., ISAYAMA, Y., KOHSAKA, Y., YAMADA, T. & TAKEO, S. (1998). Modulation of cAMP-mediated vasorelaxation by endothelial nitric oxide and basal cGMP in vascular smooth muscle. *J Cardiovasc Pharmacol*, **32,** 543-51.

TROCHU, J.N., LEBLAIS, V., RAUTUREAU, Y., BEVERELLI, F., LE MAREC, H., BERDEAUX, A. & GAUTHIER, C. (1999). Beta 3-adrenoceptor stimulation induces vasorelaxation mediated essentially by endothelium-derived nitric oxide in rat thoracic aorta. *Br J Pharmacol*, **128,** 69-76.

TSUJIMOTO, G., LEE, C.H. & HOFFMAN, B.B. (1986). Age-related decrease in beta adrenergic receptor-mediated vascular smooth muscle relaxation. *J Pharmacol Exp Ther*, **239,** 411-5.

TUNG, L.H., RAND, M.J. & MAJEWSKI, H. (1981). Adrenaline-induced hypertension in rats. *Clin Sci (Lond)*, **61 Suppl 7,** 191s-193s.

UCHIDA, E., BOHR, D.F. & HOOBLER, S.W. (1967). A method for studying isolated resistance vessels from rabbit mesentery and brain and their responses to drugs. *Circ Res*, **21,** 525-36.

UHRENHOLT, T.R., DOMEIER, T.L. & SEGAL, S.S. (2007). Propagation of calcium waves along endothelium of hamster feed arteries. *Am J Physiol Heart Circ Physiol*, **292,** H1634-40.

URECH, E., MARXER, A. & MIESCHER, K. (1950).

VAN RIJEN, H.V., VAN VEEN, T.A., HERMANS, M.M. & JONGSMA, H.J. (2000). Human connexin40 gap junction channels are modulated by cAMP. *Cardiovasc Res*, **45,** 941-51.

VANBAVEL, E., MOOIJ, T., GIEZEMAN, M.J. & SPAAN, J.A. (1990). Cannulation and continuous cross-sectional area measurement of small blood vessels. *J Pharmacol Methods*, **24,** 219-27.

VANBAVEL, E., VAN DER MEULEN, E.T. & SPAAN, J.A. (2001). Role of Rho-associated protein kinase in tone and calcium sensitivity of cannulated rat mesenteric small arteries. *Exp Physiol*, **86,** 585-92.

VENARDOS, K., ENRIQUEZ, C., MARSHALL, T., CHIN-DUSTING, J.P., AHLERS, B. & KAYE, D.M. (2009). Protein kinase C mediated inhibition of endothelial L-arginine transport is mediated by MARCKS protein. *J Mol Cell Cardiol*, **46,** 86-92.

VEQUAUD, P. & THORIN, E. (2001). Endothelial G protein beta-subunits trigger nitric oxide-but not endothelium-derived hyperpolarizing factor-dependent dilation in rabbit resistance arteries. *Circ Res*, **89,** 716-22.

VEZZA, R., HABIB, A., LI, H., LAWSON, J.A. & FITZGERALD, G.A. (1996). Regulation of cyclooxygenases by protein kinase C. Evidence against the importance of direct enzyme phosphorylation. *J Biol Chem*, **271,** 30028-33.

VOGALIS, F., HARVEY, J.R. & FURNESS, J.B. (2003). PKA-mediated inhibition of a novel K+ channel underlies the slow after-hyperpolarization in enteric AH neurons. *J Physiol*, **548,** 801-14.

WALDRON, G.J. & COLE, W.C. (1999). Activation of vascular smooth muscle K+ channels by endothelium-derived relaxing factors. *Clin Exp Pharmacol Physiol*, **26,** 180-4.

WALDRON, G.J. & GARLAND, C.J. (1994). Contribution of both nitric oxide and a change in membrane potential to acetylcholine-induced relaxation in the rat small mesenteric artery. *Br J Pharmacol*, **112,** 831-6.

WANG, Y., ZHANG, M., LIU, Y., LIU, Y. & CHEN, M. (2010). The effect of nebivolol on asymmetric dimethylarginine system in spontaneously hypertension rats. *Vascul Pharmacol*.

WANSTALL, J.C., JEFFERY, T.K., GAMBINO, A., LOVREN, F. & TRIGGLE, C.R. (2001). Vascular smooth muscle relaxation mediated by nitric oxide donors: a comparison with acetylcholine, nitric oxide and nitroxyl ion. *Br J Pharmacol*, **134,** 463-72.

WEISS, D.N., PODBERESKY, D.J., HEIDRICH, J. & BLAUSTEIN, M.P. (1993). Nanomolar ouabain augments caffeine-evoked contractions in rat arteries. *Am J Physiol*, **265,** C1443-8.

WENZEL, D., KNIES, R., MATTHEY, M., KLEIN, A.M., WELSCHOFF, J., STOLLE, V., SASSE, P., ROLL, W., BREUER, J. & FLEISCHMANN, B.K. (2009). beta(2)-adrenoceptor antagonist ICI 118,551 decreases pulmonary vascular tone in mice via a G(i/o) protein/nitric oxide-coupled pathway. *Hypertension*, **54,** 157-63.

WESTON, A.H., ABSI, M., WARD, D.T., OHANIAN, J., DODD, R.H., DAUBAN, P., PETREL, C., RUAT, M. & EDWARDS, G. (2005). Evidence in favor of a calcium-sensing receptor in arterial endothelial cells: studies with calindol and Calhex 231. *Circ Res*, **97,** 391-8.

WESTON, A.H., PORTER, E.L., HARNO, E. & EDWARDS, G. (2010). Impairment of endothelial SK(Ca) channels and of downstream hyperpolarizing pathways in mesenteric arteries from spontaneously hypertensive rats. *Br J Pharmacol*, **160,** 836-43.

WHALEN, E.J., BATES, J.N., JOHNSON, A.K. & LEWIS, S.J. (2006). Downregulation of propranolol-sensitive beta-adrenoceptor signaling after inhibition of nitric oxide synthesis. *Br J Pharmacol*, **147,** 755-64.

WHITE, R., BOTTRILL, F.E., SIAU, D. & HILEY, C.R. (2001). Protein kinase A-dependent and -independent effects of isoproterenol in rat isolated mesenteric artery: interactions with levcromakalim. *J Pharmacol Exp Ther*, **298,** 917-24.

WHITE, R.E. & CARRIER, G.O. (1986). Alpha 1- and alpha 2-adrenoceptor agonist-induced contraction in rat mesenteric artery upon removal of endothelium. *Eur J Pharmacol*, **122,** 349-52.

WHITE, R.E., LEE, A.B., SHCHERBATKO, A.D., LINCOLN, T.M., SCHONBRUNN, A. & ARMSTRONG, D.L. (1993). Potassium channel stimulation by natriuretic peptides through cGMP-dependent dephosphorylation. *Nature*, **361,** 263-6.

WINTER, P. & DORA, K.A. (2007). Spreading dilatation to luminal perfusion of ATP and UTP in rat isolated small mesenteric arteries. *J Physiol*, **582,** 335-47.
WONG, M.S., MAN, R.Y. & VANHOUTTE, P.M. (2010a). Calcium-independent phospholipase A(2) plays a key role in the endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Am J Physiol Heart Circ Physiol*, **298,** H1260-6.
WONG, S.L., WONG, W.T., TIAN, X.Y., LAU, C.W. & HUANG, Y. (2010b). Prostaglandins in action indispensable roles of cyclooxygenase-1 and -2 in endothelium-dependent contractions. *Adv Pharmacol*, **60,** 61-83.
WOOTEN, M.W., SEIBENHENER, M.L., MATTHEWS, L.H., ZHOU, G. & COLEMAN, E.S. (1996). Modulation of zeta-protein kinase C by cyclic AMP in PC12 cells occurs through phosphorylation by protein kinase A. *J Neurochem*, **67,** 1023-31.
WU, C.C., CHEN, S.J. & YEN, M.H. (1997). Loss of acetylcholine-induced relaxation by M3-receptor activation in mesenteric arteries of spontaneously hypertensive rats. *J Cardiovasc Pharmacol*, **30,** 245-52.
XAVIER, F.E., BLANCO-RIVERO, J., FERRER, M. & BALFAGON, G. (2009). Endothelium modulates vasoconstrictor response to prostaglandin I2 in rat mesenteric resistance arteries: interaction between EP1 and TP receptors. *Br J Pharmacol*, **158,** 1787-95.
XIA, X.M., ZENG, X. & LINGLE, C.J. (2002). Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature*, **418,** 880-4.
XIONG, Z., SPERELAKIS, N. & FENOGLIO-PREISER, C. (1994). Regulation of L-type calcium channels by cyclic nucleotides and phosphorylation in smooth muscle cells from rabbit portal vein. *J Vasc Res*, **31,** 271-9.
XU, C., LU, Y., TANG, G. & WANG, R. (1999). Expression of voltage-dependent K^+ channel genes in mesenteric artery smooth muscle cells. *Am. J. Physiol. Gas Liv Phy*, **277,** G1055-1063.
XU, K.M., TANG, F. & HAN, C. (1997). Alterations of mRNA levels of alpha 1-adrenoceptor subtypes with maturation and ageing in different rat blood vessels. *Clin Exp Pharmacol Physiol*, **24,** 415-7.
XU, S.Z. & BEECH, D.J. (2001). TrpC1 is a membrane-spanning subunit of store-operated Ca(2+) channels in native vascular smooth muscle cells. *Circ Res*, **88,** 84-7.
XU, S.Z., BOULAY, G., FLEMMING, R. & BEECH, D.J. (2006). E3-targeted anti-TRPC5 antibody inhibits store-operated calcium entry in freshly isolated pial arterioles. *Am J Physiol Heart Circ Physiol*, **291,** H2653-9.
XU, Z., YI, Z., DANG, X., WU, X., CAO, Y., HUANG, D., MO, S. & HE, X. (2010). [Sympathetic nervous system level and ambulatory blood pressure in children with primary nephrotic syndrome.]. *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, **35,** 693-8.
YAMAMOTO, Y., IMAEDA, K. & SUZUKI, H. (1999). Endothelium-dependent hyperpolarization and intercellular electrical coupling in guinea-pig mesenteric arterioles. *J Physiol*, **514 (Pt 2),** 505-13.
YAMAMOTO, Y., KLEMM, M.F., EDWARDS, F.R. & SUZUKI, H. (2001). Intercellular electrical communication among smooth muscle and endothelial cells in guinea-pig mesenteric arterioles. *J Physiol*, **535,** 181-95.
YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332,** 411-5.

YAO, X. & GARLAND, C.J. (2005). Recent developments in vascular endothelial cell transient receptor potential channels. *Circ Res*, **97,** 853-63.
YEROMIN, A.V., ZHANG, S.L., JIANG, W., YU, Y., SAFRINA, O. & CAHALAN, M.D. (2006). Molecular identification of the CRAC channel by altered ion selectivity in a mutant of Orai. *Nature*, **443,** 226-9.
YOON, H., CHUNG, W.S., PARK, Y.Y. & CHO, I.H. (2005). Effects of stress on female rat sexual function. *Int J Impot Res*, **17,** 33-8.
YUILL, K.H., MCNEISH, A.J., KANSUI, Y., GARLAND, C.J. & DORA, K.A. (2009). Nitric Oxide Suppresses Cerebral Vasomotion by sGC-Independent Effects on Ryanodine Receptors and Voltage-Gated Calcium Channels. *J Vasc Res*, **47,** 93-107.
YUILL, K.H., YAROVA, P., KEMP-HARPER, B.K., GARLAND, C.J. & DORA, K.A. (2010). A Novel Role for HNO in Local and Spreading Vasodilatation in Rat Mesenteric Resistance Arteries. *Antioxid Redox Signal*.
ZACHARIA, I.G. & DEEN, W.M. (2005). Diffusivity and solubility of nitric oxide in water and saline. *Ann Biomed Eng*, **33,** 214-22.
ZHANG, L., DILIZIO, C., KIM, D., SMYTH, E.M. & MANNING, D.R. (2006a). The G12 family of G proteins as a reporter of thromboxane A2 receptor activity. *Mol Pharmacol*, **69,** 1433-40.
ZHANG, M., SONG, P., XU, J. & ZOU, M.H. (2010). Activation of NAD(P)H Oxidases by Thromboxane A2 Receptor Uncouples Endothelial Nitric Oxide Synthase. *Arterioscler Thromb Vasc Biol*.
ZHANG, S.L., YU, Y., ROOS, J., KOZAK, J.A., DEERINCK, T.J., ELLISMAN, M.H., STAUDERMAN, K.A. & CAHALAN, M.D. (2005). STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. *Nature*, **437,** 902-5.
ZHANG, W., ZHANG, Y., EDVINSSON, L. & XU, C.B. (2009a). Transcriptional down-regulation of thromboxane A(2) receptor expression via activation of MAPK ERK1/2, p38/NF-kappaB pathways. *J Vasc Res*, **46,** 162-74.
ZHANG, X.P. & HINTZE, T.H. (2006b). cAMP signal transduction induces eNOS activation by promoting PKB phosphorylation. *Am J Physiol Heart Circ Physiol*, **290,** H2376-84.
ZHANG, Y.H. & HANCOX, J.C. (2009b). Regulation of cardiac Na+-Ca2+ exchanger activity by protein kinase phosphorylation--still a paradox? *Cell Calcium*, **45,** 1-10.
ZHOLOS, A., JOHNSON, C., BURDYGA, T. & MELANAPHY, D. (2011). TRPM Channels in the Vasculature. *Adv Exp Med Biol*, **704,** 707-29.
ZHOU, Q.G., HU, Y., HUA, Y., HU, M., LUO, C.X., HAN, X., ZHU, X.J., WANG, B., XU, J.S. & ZHU, D.Y. (2007). Neuronal nitric oxide synthase contributes to chronic stress-induced depression by suppressing hippocampal neurogenesis. *J Neurochem*, **103,** 1843-54.
ZHOU, X., BOHLEN, H.G., MILLER, S.J. & UNTHANK, J.L. (2008). NAD(P)H oxidase-derived peroxide mediates elevated basal and impaired flow-induced NO production in SHR mesenteric arteries in vivo. *Am J Physiol Heart Circ Physiol*, **295,** H1008-H1016.
ZHU, S., WHITE, R.E. & BARMAN, S.A. (2006). Effect of PKC isozyme inhibition on forskolin-induced activation of BKCa channels in rat pulmonary arterial smooth muscle. *Lung*, **184,** 89-97.
ZWAVELING, J., WINKLER PRINS, E.A., PFAFFENDORF, M. & VAN ZWIETEN, P.A. (1996). The influence of hyperthyroidism on beta-adrenoceptor-mediated relaxation of

isolated small mesenteric arteries. *Naunyn Schmiedebergs Arch Pharmacol*, **353,** 438-44.

ZYGMUNT, P.M. & HOGESTATT, E.D. (1996). Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br J Pharmacol*, **117,** 1600-6.